Medical Library
MAR 3 1944

VOL. XXIV. No. 6] [DECEMBER, 1943

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

EDITOR
R. W. Scarff

ASSISTANT EDITORS
G. P. Gladstone, N. H. Martin, F. R. Selbie, D. D. Woods

ADVISORY EDITORS
S. P. Bedson, J. R. Marrack, Wilson Smith

with the support of

E. C. Dodds, J. C. Drummond, C. L. Evans, H. W. Florey,
W. E. Gye, H. D. Kay, J. McIntosh, J. W. McLeod,
J. A. Murray, W. J. Tulloch

DIRECTOR
Paul Fildes

LONDON
H. K. LEWIS & Co. Ltd.
136 GOWER ST., W.C. 1

CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO

on behalf of

'The British Journal of Experimental Pathology.' Registered Office: 120 Moorgate, London, E.C. 2

Annual Subscription, January to December, post free, Two Pounds net
Single Numbers, Seven and Sixpence net

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

VOL. XXIV, No. 6. DECEMBER, 1943.

CONTENTS.

OSBORN, E. M.—ON THE OCCURRENCE OF ANTIBACTERIAL SUBSTANCES IN GREEN PLANTS 227

BERENBLUM, I., AND SCHOENTAL, R.—CARCINOGENIC CONSTITUENTS OF SHALE OIL . 232

MERLING, K. B. E.—ULTRAMICROSCOPICAL OBSERVATIONS ON THE MORPHOLOGY AND DEVELOPMENT OF VACCINIA VIRUS *in vitro* 240

MCINTOSH, J., AND SELBIE, F. R.—THE PRODUCTION OF DRUG-RESISTANT CULTURES OF BACTERIA *in vitro* AND A STUDY OF THEIR INTER-RELATIONSHIPS . . 246

BERGER, F. M.—THE SENSITIZING AND INDICATOR ACTION OF VICTORIA BLUE AND JANUS GREEN ON THE FLOCCULATION REACTION FOR SYPHILIS 252

INDEX TO AUTHORS 261

SURVEY OF PAPERS i

EDITORIAL NOTICE TO CONTRIBUTORS.

Scope of Papers.—This Journal was founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, pharmacological, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects.

Forwarding Papers and Correspondence.—Papers should be forwarded to Dr. R. W. SCARFF, Middlesex Hospital, London, W. 1.

General Correspondence relating to the conduct of the Journal should be addressed to Dr. PAUL FILDES, Middlesex Hospital, W. 1.

Subscriptions and business correspondence are received by the publishers, Messrs. H. K. Lewis & Co. Ltd., 136, Gower Street, W.C. 1.

Date of Publication.—The Journal is published bi-monthly, in the middle of February, April, June, August, October, and December.

Rapidity of Publication.—All papers accepted for publication will be printed, so far as possible, in the next following issue of the Journal, if received before the middle of the month preceding publication. Long papers, or papers with illustrations, may, however, be delayed if not received earlier.

Proofs.—To avoid delay, proofs will not be submitted to Authors overseas unless they express a desire to see them, or unless the Editors think it necessary. Overseas Authors are therefore particularly reminded of the importance of preparing their manuscripts in such a way that error need not arise.

Reprints.—25 reprints will be supplied free to authors who are registered as personal subscribers to the Journal. Additional copies must be ordered from the Publishers *before the issue is printed*. Prices on application.

Made and printed in Great Britain.

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XXIV DECEMBER, 1943 NO. 6

ON THE OCCURRENCE OF ANTIBACTERIAL SUBSTANCES IN GREEN PLANTS.

E. M. OSBORN.

From the Sir William Dunn School of Pathology, University of Oxford.

Received for publication August 28, 1943.

FROM earliest times plants have been used in an attempt to cure disease. Claims have been made that certain plants have the power of healing wounds and removing inflammation, and that others are effective in the treatment of such diseases as cholera and dysentery. It might be assumed that if these observations were true the cures claimed may have been influenced in some cases by the presence of antibacterial substances in the plants used.

In recent literature the bactericidal effects of substances contained in certain green plants have been described. Jordinoff (1927) demonstrated the bactericidal action of *Capsicum annuum* on *Bact. typhosum* and other pathogenic organisms. Stickl (1928) showed that extracts of *Chelidonium majus* and some of its constituents were bactericidal against staphylococci and anthrax organisms. Boes and Stende (1935) and Keding (1939) observed the bactericidal effect of anemonin, a volatile substance contained in *Ranunculus acris*, while Valette and Liber (1938) showed that convolvulin had a bactericidal effect on pneumococci. Thorne and Browne (1937), studying the growth and respiration of soil bacteria in juices of leguminous and non-leguminous plants, found that most of the leguminous bacteria studied were able to grow in the juice of their host plant, but that those juices were bactericidal to other species of root nodule bacteria. Foter and Golich (1938) and Foter (1940), using crushed horse radish, demonstrated the bactericidal action of the contained allyl isothiocyanate and related mustard oils. Bocker (1938) compared the sterilizing effect of garlic vapours on staphylococci under varying conditions. Sherman and Hodge (1936) demonstrated the bactericidal action of the raw juices expressed from cabbages and turnips.

Though the presence of antibacterial substances in green plants is well known, no systematic search for such substances has apparently yet been made.

The present paper contains the first results of such an investigation. Approximately 2300 different green plants belonging mainly to the group of angiosperms (flowering plants) have been tested.

EXPERIMENTAL.

Method.—In selecting a method for testing for bacterial inhibition a number of factors have to be borne in mind. The method must be relatively simple and should require a small amount of material, since only small amounts of some of the plants

TABLE I.

Explanation of symbols used.

\+ = a complete inhibition of 10–20 mm. diam.
++ = „ „ 20–30 mm. „
+++ = „ „ 30–40 mm. „
++++ = „ „ 40–50 mm. „
+++++ = „ „ 50–60 mm. „
++++++ = „ „ 50–70 mm. „

(S) = extract made from seeds only; (X) = garden hybrid.

	Staph. aureus.	*B. coli.*
Amaryllidaceae.		
Alstroemeria aurantica Don	+	++
„ *haemantha* Ruiz & Pav.	+	++
Annonaceae.		
Annona cherimolia Mill.	++	++
Artabotrys odoralissimus Blume	+++	+++
Mischogyne michelioides Exell	++	++
Apocynaceae.		
Allamanda cathartica L.	+	..
„ „ var. *hendersonii* Hort.	+	..
„ *neriifolia* Hook.	+	..
Plumeria bicolor Ruiz & Pav.	+	..
Araliaceae.		
Dendropanax japonicum Seem.	+	..
Aristolochiaceae.		
Aristolochia clematitis L.	+	..
„ *fimbriata* Cham.	++	..
Asarum canadense L.	+	..
„ *europaeum* L.	+	..
Berberidaceae.		
Epimedium peraldianum Coss.	+	..
„ *pinnatum* Fisch.	+	..
(X) „ *youngianum* Fisch. & Mey. var. *niveum*	+	..
Mahonia fortunei Fedde	+	..
Capparidaceae.		
Steriphoma ellipticum Spreng.	+	..
„ *paradoxum* Endl.	+	..
Compositae.		
Arctium lappa L.	+	..
Arctotis stoechadifolia Berg.	+	..
Candidea stenostegia Stapf	+	..
Centaurea macrocephala Puschk.	+	..
„ *pulchra* DC.	++	..
„ *scabiosa* L.	+	..
Crepis capillaris (L.) Wallr.	+	..
„ *incana* Sibth. & Sm.	+	..
„ *tarazacifolia* Thuill.	+	..
Hieracium amplexicaule L.	+	..
„ *aurantiacum* L.	+	..
„ *gymnocephalum* Griseb.	+	..
„ *murorum* L.	+	..
„ *rupestre* All.	+	..
„ *umbellatum* L.	+	..
Lactuca plumieri Gren. & Godr.	+	..
Lapsana communis L.	+	..
Sonchus palustris L.	+	..
Stifftia chrysantha Mikan.	+	..
Tanacetum vulgare L.	+	..
„ „ var. *crispum* DC.	+	..

	Staph. aureus.	*B. coli.*
Cruciferae.		
Aethionema pulchellum Boiss.	+	..
Brassica oleracea L.: The following commercial varieties—		
(S) Savoy cabbage, "Best of All"	++	++
(S) Cabbage, "Prince Nonpareil"	+	+
(S) Red cabbage	++	++
(S) "Giant Cauliflower"	++	++
(S) "Late Queen Broccoli"	++	++
(S) "Purple Sprouting Broccoli"	++	+++
(S) "White Sprouting Broccoli"	+	+
(S) Borecole, "Dwarf Green Curled"	+	?
(S) Brussels sprouts, "Wroxton"	+	+
(S) Kohl rabi	++	++
(Extracts of the vegetative parts of the above varieties of *Brassica oleracea* produced a slight partial on *Staph. aureus* only.)		
(S) *Cheiranthus allionii* Hort.	++	++
„ *cheiri* L.	+	+
(S) *Erysimum perofskianum* Fisch. & Mey.	++	++
(S) *Iberis sempervirens* L.	++	+++
„ L. "Superba"	+	+
Lunaria annua L.	+	+
(S) *Malcolmia maritima* R. Br.	+	++
(S) *Matthiola bicornis* DC.	+	+
„ *incana* R. Br.	+	+
(S) „ „ „ var. *annua* Voss., "ten-week stock"	++	++
Datiscaceae.		
Datisca cannabina L.	+	..
Ericaceae.		
Arbutus canariensis Duham.	+	+
Flacourtiaceae.		
Azara arborea L.	+	+
„ *gilliesii* Hook. & Arn.	+	+
„ *integrifolia* Ruiz & Pav.	+	+
„ *microphylla* Hook. f.	+	+
Flacourtia cataphracta Roxb.	..	+
„ *ramoutchi* L'Hérit.	+	+
Gynocardia odorata Roxb.	++	+++
Hydnocarpus anthelminticus Pierre	++	++
„ *ilicifolius* King	+++	+++
„ *wightianus* Blume	+	++
Taraktogenos kurzii King	++	+++

TABLE I—*continued.*

	Staph. aureus.	*B. coli.*
Gnetaceae.		
Gnetum gnemon L.	..	+
Guttiferae.		
Hypericum elodes L.	+	..
,, *olympicum* L.	+	..
Labiatae.		
(X) *Coleus blumei* Benth.	+	..
,, *thyrsoideus* Baker	+	..
Leguminoseae.		
Bauhinia hawkesiana F. M. Bailey	+	+
Liliaceae.		
Allium sativum L.	+++	++
,, *triquetrum* L.	+	++
,, *ursinum* L.	+	+
Erythronium dens-canis L.	++	++
,, *tuolmnensis* Applegate	+	++
Tulipa chrysantha Boiss.	++	++
,, *clusiana* DC.	++	++
,, *cretica* Boiss. & Heldr.	++	++
,, *forsteriana* Regel	++	++
,, *gesneriana* L.	++	++
,, *greigii* Regel	++	++
,, *Kaufmanniana* Regel	+	+
,, *kolpatowskyana* Regel	+	+
,, *linifolia* Regel	++	+
,, *turkestanica* Regel	++	++
Magnoliaceae.		
Magnolia acuminata L.	+	..
,, *grandiflora* L.	+	..
Michelia fuscata Blume	+	..
Moraceae.		
Dorstenia barteri Don	+	+
,, *elata* Gardn.	+	++
,, *mannii* Hook. f.	+	+
Oleaceae.		
Jasminum rex Dunn	+	..
Ligustrum nepalense Wall	+	..
,, *vulgare* L.	+	..
,, *Walkeri* Decne.	+	..
Olea capensis L.	+	..
Papaveraceae.		
Corydalis bulbosa Sw.	+	..
,, *lutea* DC.	+	..
Passifloraceae.		
Ophiscaulon cissampeloides Hook. f.	+	..
Passiflora suberosa L.	++	++
Psilotaceae.		
Psilotum triquetrum Sw.	+	+
Ranunculaceae.		
Anemone apennina L.	++++	++++
,, *pulsatilla* L.	++++	+++
,, *rupicola* Camb.	+++	++
Clematis armandii Franchet	+++	+
,, *fremontii* S. Wats.	++++	+++
,, *heracleaefolia* DC. var. *davidiana*	++++	+++
(S) ,, *recta* L.	++++++	++++++
,, *stans* Sieb. & Zucc.	++++	++
,, *texensis* Buckl.	+++	+++
Helleborus niger L.	++++	+++
,, *viridis* L.	++++	++++
Ranunculus acris L.	++++	++
,, *bulbosus* L.	+++	++
,, *flammula* L.	++++	++
,, *lingua* L.	++++	+++
Resedaceae.		
Reseda lutea L.	++	..
Rhamnaceae.		
Rhamnus catharticus L.	+	..
Rosaceae.		
Spiraea aruncus L.	+	++
,, *bullata* Maxim	+	..
(X) ,, *bumalda* Burr.	+	+
,, *japonica* L., f. var. *alba* Hort.	+	++
,, *thunbergii* Sieb. & Zucc.	++	++
Sapindaceae.		
Cardiospermum halicacabum L.	+	+

The table lists only those species which give frankly positive results. By kind permission of the Council of the Royal Society of London a full list of species tested to the end of October, 1943, with the results obtained, has been lodged in the archives of the Society at Burlington House, London. Copies of this list may be obtained on application to the Sir William Dunn School of Pathology, Oxford.

to be tested are available. These two considerations alone are felt to be heavily in favour of the diffusion method (Abraham *et al.*, 1940) as compared to dilution methods, where Seitz filtration or some other process for obtaining a sterile extract is required. The drawbacks of the diffusion method are (1) it is not quite as sensitive as the dilution test, (2) inhibitors which will not diffuse through agar will give a negative result, and (3) the test is only qualitative (unless conditions under which it can be used quantitatively, e.g. as for penicillin, are worked out). With regard to the second of these drawbacks, an inhibitor which will not diffuse through agar would probably in any case be held back by a Seitz filter; the first and third objections are felt to be of minor importance in a general survey of this nature. In view of the first objection it is not surprising that certain materials which have been found by other workers to have a mild bactericidal action have here not given positive results (see below).

Procedure.—All experiments were carried out in the first place with freshly picked material.

In testing a given plant it was found desirable to use portions of all available parts —roots, stem, leaf and reproductive organs. In certain cases the inhibitory substance was found to be produced by an enzyme acting on an inactive precursor, the enzyme being located in one part of the plant, the precursor in another; unless both parts were used together in the test no inhibitor was produced.

The material was ground up by pestle and mortar with sand and sufficient distilled water to produce a pulp. This was strained through fine silk, and the resulting extract tested against *Staphylococcus aureus* and *Bact. coli* by a method previously described by Abraham *et al.* (1940).

The result obtained by the above method is qualitative; it merely shows the presence or absence of antibacterial substances in a given extract. A negative result does not necessarily prove absence of such substances in the plant concerned, for it is recognized that the method of preparation may not reveal all antibacterial substances. Also the possibility of enzymic destruction should be borne in mind.

Further tests were made with some plants giving positive results to determine (1) the distribution of the active substance within the plant, (2) whether it is produced as a result of enzyme activity, and (3) to test its stability during wilting and drying of the plant.

RESULTS.

Of the plants tested to date 63 genera belonging to 28 families gave positive results. Details are shown in the Table.

The inhibitory substances vary in specificity and potency. Some inhibit the growth of both test organisms, while others are specific against *Staph. aureus*. Under the conditions of the test used the extracts of two plants are specific against *Bact. coli*.

It will be seen from the results in the Table that the specificity and potency of extracts of plants of one family tend to be similar throughout the family, suggesting that similar types of antibacterial substances occur in those species of the family. For example, extracts of plants belonging to the family Ranunculaceae are active against both test organisms, and are remarkable for the large zones of inhibition produced; while in the family Compositae extracts of all active species found to date are specific against *Staph. aureus*.

The distribution of the active substance in the plant varies in different cases; in many, e.g. *Asarum europaeum*, it would seem to be contained in all parts of the plant; in others, the concentration in one part of the plant, e.g. in the seeds of *Brassica oleracea*, greatly exceeds that in any other part; whilst in some cases the active substance is restricted to one part, e.g. the bark in *Magnolia acuminata*.

In many plants, e.g. *Plumeria bicolor*, *Cheiranthus cheiri*, *Crepis taraxacifolia* and others, the inhibitory substances are produced as a result of enzyme action.

The degree of stability of the active substances during the wilting and drying of the plant also varies greatly. Dried material of *Crepis taraxacifolia*, *Reseda lutea*, *Asarum europaeum* and others retains full inhibitory power after being kept for one year; other plants begin to lose the power of inhibition in two days after picking and when fully dried give negative results—examples are *Ranunculus*, *Clematis*, *Anemone* (Ranunculaceae), *Gynocardia* and *Hydnocarpus* (Flacourtiaceae).

Certain plants recorded by other workers as causing bactericidal action have here failed to produce results. This applies to *Chelidonium majus* (Stickl, 1928), turnip (Sherman and Hodge, 1936), and horse radish (Foter, 1940). Disparity in results in these cases is probably due partly to differences in the methods used, described above, and also to the part or parts of the plant used in making the extract. Sherman and Hodge (1936), using the juice expressed from heads of cabbages, record "a relatively

mild but distinct bactericidal action." Tested by the method used here extract of cabbage heads causes only a slight partial inhibition of *Staph. aureus* which is scarcely measurable, but extract of seeds of cabbage causes complete inhibition of both test organisms.

Certain well-known drug plants, e.g. *Atropa*, *Datura* and *Digitalis*, show no inhibitory power when tested by this method; the same applies to many plants recommended by the herbalists.

SUMMARY.

1. Approximately 2300 species of plants belonging to 166 families have been tested against *Staph. aureus* and *Bact. coli*. Of these, 63 genera contain substances which inhibit the growth of one or both test organisms.

2. Extracts made from plants belonging to the same family tend to show a similar specificity and potency.

3. Inhibitory substances are in some cases distributed throughout the plant, in others restricted to one part.

4. Drying of some plants causes a loss of inhibitory power; in other cases there is no loss.

5. Certain well-known drug plants tested by this method show no inhibitory power.

For frequent supplies of material I am indebted to the Director, Royal Botanic Garden, Kew; to the Regius Keeper, Royal Botanic Garden, Edinburgh; to the Curators of the Botanic Garden, Oxford; to the Keeper of the Groves, St. John Baptist College, Oxford; and to Dr. Wells, Shipton Manor, Oxford.

This survey was suggested by Prof. Florey and Dr. Chain. I wish to offer them, and also Dr. Heatley of this Department, my thanks for help during the progress of the work. I am indebted to Dr. A. R. Clapham, Botanical Department, Oxford, for advice on taxonomic questions.

REFERENCES.

Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G., and Jennings, M. A.—(1941) *Lancet*, ii, 177.

Boas, F., and Steude, R.—(1935) *Biochem. Z.*, **279**, 417.

Böcker, O. E.—(1938) *Z. Hyg. Infektkr.*, **121**, 166.

Foter, M. J., and Golick, Ann M.—(1938) *Food Res.*, **3**, 609 (quoted from *Biol. Abstr.* (1939), **13**, 266).

Foter, M. J.—(1940) *Ibid.*, **5**, 147 (quoted from *Biol. Abstr.* (1940), **14**, 1176).

Jordanoff, M.—(1927) *Jber. Univ. Sofia, Vet. Med. Facultät.*, **3**, 55 (quoted from *Biol. Abstr.* (1931), **5**, 2379).

Keding, F.—(1939) *Angew. Bot.*, **21**, 1.

Sherman, J. M., and Hodge, H. M.—(1936) *J. Bact.*, **31**, 96.

Stickl, O.—(1928) *Z. Hyg. Infektkr.*, **108**, 566.

Thorne, D. W., and Brown, P. E.—(1937) *J. Bact.*, **34**, 567.

Valette, G., and Liber, A.—(1938) *C. R. Soc. Biol.*, **128**, 362.

CARCINOGENIC CONSTITUENTS OF SHALE OIL.

I. BERENBLUM AND R. SCHOENTAL.

From the Oxford University Research Centre of the British Empire Cancer Campaign, Sir William Dunn School of Pathology, The University of Oxford.

Received for publication September 10, 1943.

OF the various mineral oils tested for carcinogenic action, Scottish shale oil is known to be the most potent (Twort and Lyth, 1933). The carcinogenicity of shale oil was originally demonstrated experimentally by Leitch (1922), who found, moreover, that the blue fraction of shale oil was particularly potent.

Attempts have been made, by fractional distillation and extraction, to discover the nature of the substances responsible for the carcinogenic action of mineral oils. Similar investigations on coal-tar led to the isolation and identification of a potent carcinogen—3 : 4-benzpyrene (Hieger, 1930 ; Cook, Hewett and Hieger, 1933). The results with mineral oils (Twort and Ing, 1928 ; Twort and Fulton, 1929, 1932 ; Twort and Lyth, 1939) were disappointing ; no carcinogenic constituents were isolated or identified, and little evidence was obtained as to the class of compounds to which they belonged ; moreover, the results obtained were in many cases conflicting, and this gave the impression that destruction of the active constituents may have occurred during the experimental procedure. More recent reports (*B.E.C.C. Rep.*, 1940, 1941, 1942) refer to the use of chromatography in conjunction with the methods previously used, by which Twort and his co-workers claim the isolation of some unidentified crystalline hydrocarbons from shale oil. But these seemed to be non-carcinogenic.

In the present investigation, chromatography, in conjunction with fluorescence spectrography, served as the principal methods of fractionation of blue shale oil, the highly carinogenic fraction technically obtained during the refining of shale oil.

METHODS.

The alumina used for chromatography was "Aluminium oxide for adsorption purposes," B.D.H. Ltd. Since large amounts were required, the alumina was recovered several times and used over again. (For the more exacting liquid chromatography, only reactivated new alumina was used.) The recovery was by washing out with ethanol as much as possible of the adsorbed material, and then incineration for several hours at about 500° C. in an electric muffle furnace. (Soon after incineration, the adsorbing power of the alumina was excessively high, but after being kept for several days it approached that of the original alumina.)

While biological tests served as the ultimate criterion for carcinogenesis, an additional, more rapid means of gauging successful fractionation was required. In the case of coal-tar a study of fluorescence spectra proved invaluable for this purpose (Hieger, 1930, 1937 ; Cook *et al.*, 1933), and though some controversy arose as to the specificity of fluorescence spectra in mixtures of hydrocarbons (Sannié, 1936), more

recent studies (Mayneord and Roe, 1936 ; Hieger, 1937 ; Miller and Baumann, 1943) have vindicated its usefulness. The method was found very effective in the present investigation.

The method used for obtaining fluorescence spectra was based on that originally described by Hieger (1930), except that a Bellingham & Stanley quartz spectrograph was used, having a somewhat larger dispersion than the Hilger spectrograph No. E31 used by Hieger. The spectra were photographed on Kodak spectrographic plate I-H (sensitivity to about 560 mμ.) and I-F (to about 690mμ.). The different products, in varying concentrations in light petroleum, were tested in thin-walled Monax tubes (7–8 mm. diam.). Exposures ranged from 1–5 minutes.

For carcinogenicity tests, white mice (bred in the Department from a stock originally derived from a single litter) were used. Applications to the skin (dorsal region of the back) were made at half-weekly intervals. About 10 mice were used for each fraction, and each experiment continued for 18–20 weeks. This was considered sufficient, since the object was to discover which were the highly potent fractions, not the relative carcinogenicity of weaker fractions.

The crude oil, and the liquid fractions derived from it, were applied undiluted, or, when very viscous, only slightly diluted with benzene. The solid fraction (OP) was used as a saturated solution in benzene.

EXPERIMENTAL.

Carcinogenicity of Fractions of Shale Oil obtained by Chromatography.

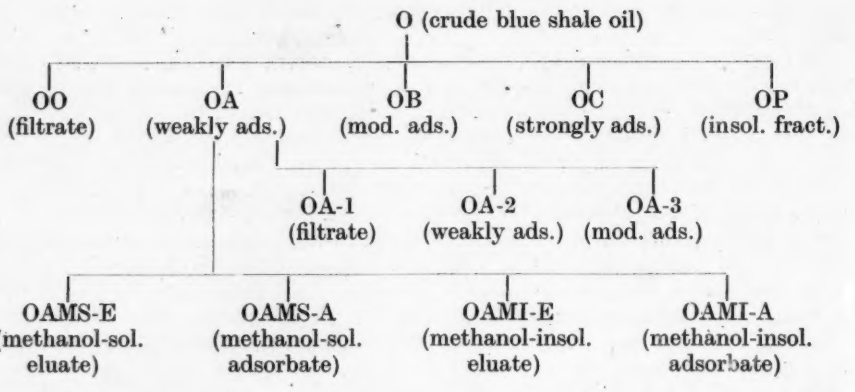
About 250 ml. of blue shale oil was mixed with 10 volumes of light petroleum (B.P. 60–80° C.), and the dark brown precipitate formed was separated by filtration (fraction OP). The filtrate was passed through several large columns of alumina (15–20 cm. long, 7–8 cm. wide). In each case, when the colourless filtrate became greenish, further addition of the solution was discontinued, and the column was developed with light petroleum. The columns were then cut according to the appearance of the predominant zones : a lower (greenish yellow, merging into orange yellow), an intermediate (brick red), and an upper (greyish brown) zone. Each of these was separately eluted with acetone, the latter distilled off under reduced pressure, and the residue dissolved in light petroleum. The chromatography of each fraction was repeated, and, after combining corresponding samples, the following fractions were obtained : Fraction OO = colourless filtrate ; OA = orange ; OB = reddish brown ; and OC = dark brown (top fraction).

These four fractions, as well as insoluble fraction OP, and a sample of whole oil (fraction O), were tested for carcinogenic action on mouse's skin. The results showed (Table I) that whole oil (O) and fraction OA were strongly carcinogenic ; OB fairly strongly, OC weakly, and OO and OP not carcinogenic at all.

The chromatography of fraction OA was then repeated with more prolonged development than previously. Three fractions were thus obtained : A filtrate (OA-1), green in colour (containing, in addition to the green portion of OA, some colourless liquid, probably belonging to OO) ; a weakly adsorbed fraction (OA-2), orange in colour ; and an upper fraction (OA-3), reddish brown in colour (probably containing some parts of OB). Tests on mouse's skin indicated (Table I) that the carcinogenic constituents were almost entirely confined to fraction OA-2. (By subcutaneous injection, however, no tumours were obtained with this fraction.)

A different fractionation of OA was attempted by a combination of extraction and chromatography. The product was repeatedly extracted with methanol, and both the methanol-soluble fraction (OAMS) and the methanol-insoluble fraction (OAMI)

TABLE I.



Fraction.*	Carcinogenicity.†	Benzpyrene flourescence bands.
O	+++++ (7½ weeks ; 6/10)	? trace
OO	— (— ; 0/6)	—
OA	+++++ (6½ weeks ; 5/9)	++
OB	++++ (11 „ ; 3/8)	+
OC	+ (16 „ ; 1/6)	—
OP	— (— ; 0/7)	—
OA-1	— (— ; 0/6)	—
OA-2	+++++ (10 weeks ; 5/8)	—
OA-3	+ (16 „ ; 1/6)	+++
OAMS-E	— (— ; 0/10)	? trace
OAMS-A	— (— ; 0/10)	„
OAMI-E	++++ (14 weeks ; 3/10)	+
OAMI-A	++ (15 „ ; 1/10)	++

* Fractions OAMS-E, OAMS-A, OAMI-E and OAMI-A applied to the mouse's skin once weekly. All other fractions applied twice weekly.

† The figures in brackets indicate the time of appearance of the first tumour in the series, and the number of survivors bearing tumours at the end of the experiment.

were divided by chromatography into an adsorbate (OAMS-A and OAMI-A respectively) and an eluate (OAMS-E and OAMI-E respectively). The results (Table I) were not sufficiently promising to warrant further fractionation on these lines.

Fluorescence Spectra of Chromatographic Fractions of Blue Shale Oil.

In his detailed study of the spectra of cancer-producing tars and oils and related substances, Hieger (1930) found that blue shale oil, refined lubricating oil, and unheated Californian petroleum, gave diffuse fluorescence spectra covering approximately the whole range of 400–440 mμ., without differentiation into bands. In this respect they differed from most tars and tar fractions which exhibited banded spectra.

Preliminary tests of the crude blue shale oil used in the present investigation also gave a diffuse fluorescence spectrum, though with some slight banding discernible. However, after fractionation by chromatography, distinct banded spectra were exhibited, especially by fractions OA and OB. Moreover, of the subsequent fractiona-

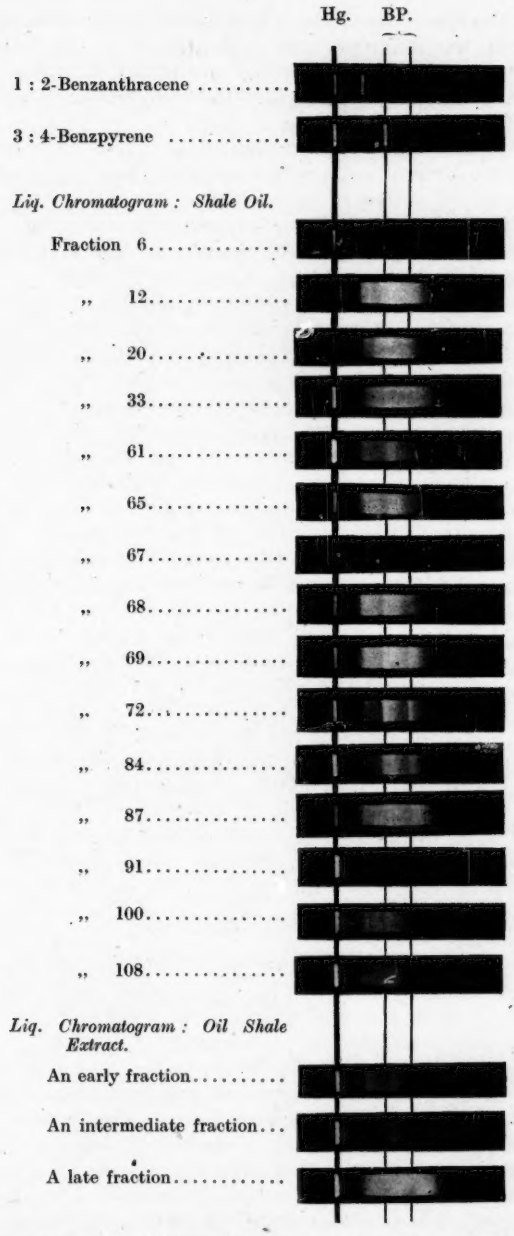


FIG. 1.—Fluorescence spectra of 1 : 2-benzanthracene, 3 : 4-benzpyrene, and fractions obtained from shale oil and from oil shale extract by chromatography. The lines marked BP represent the positions of the two most prominent fluorescence bands of benzpyrene (in light petroleum). These are approximately at 404 and 427 mμ.

tion products of OA, the spectrum of OA-1 was diffuse, while those of OA-2 and OA-3 were distinctly banded, but different from each other: In OA-3 the bands of 3 : 4-benzpyrene, both in respect of their positions and fluted structure, were clearly distinguishable, while these particular bands were absent in the more carcinogenic fraction OA-2.

A more detailed chromatography was then undertaken. A solution of 10 ml. of blue shale oil in about a litre of light petroleum was passed through a large column (20 cm. long, 6 cm. wide) of activated new alumina, the ratio of alumina to oil being, therefore, about 10 times higher than in the previous fractionations. The column was developed with large amounts of light petroleum, followed by mixtures of this and benzene, with increasingly higher proportions of the latter, and finally with pure benzene, and the eluates were collected as separate successive samples of 200–500 ml. each. The development was continued for about a week, by which time 110 separate fractions of the liquid chromatogram had been collected. (In concentrated samples of some of these fractions crystalline deposits tended to separate out on slow evaporation.)

Further development was not considered necessary, since the strongly adsorbed substances still remaining on the column (representing the higher parts of fraction OC) were known to have only a diffuse spectrum and to be non-carcinogenic.

Fluorescence spectra of all the samples collected were examined photographically, some representative examples of which are shown in Fig. 1. Among the early samples a band system not unlike that of 1 : 2-benzanthracene was present. This was followed later by the characteristic bands of 3 : 4-benzpyrene (see fractions 68–84, Fig. 1). In later fractions still other band systems were present, some of which seemed similar to those seen in comparable chromatographic fractions of coal-tar (see *B.E.C.C. Rep.*, 1943). Their identity has not yet been established. (The spectrum of 3 : 4-benzpyrene is more distinctive, and therefore more easily recognizable, than that of most other hydrocarbons, not only from the positions of the bands, but also because of the general pattern, e.g. the narrowness and reduplication of the first two bands, and also because of the exceptional intensity of the fluorescence in high dilution.)

Fractions 68–85 (i.e. those exhibiting benzpyrene bands) were recombined, concentrated under reduced pressure, and made up to a known volume (250 ml.). A part of this was kept for quantitative estimation (see below). The remainder was submitted again to a liquid chromatogram, and those fractions from it showing the strongest benzpyrene bands were again combined, evaporated, and the residue sublimed in high vacuum (at about 10^{-3} mm. Hg.). The yellow sublimate coming over at 100–120° C. exhibited intense benzpyrene fluorescence bands, but still accompanied by another band system.

Since neither repeated chromatography nor high vacuum sublimation proved adequate for the isolation of benzpyrene in a pure state, and since its isolation and identification by more orthodox methods had to be excluded on practical grounds,* identification by indirect methods was attempted.

The fraction, suspected of containing benzpyrene, was oxidized with chromic acid. The orange-coloured product thus obtained was dissolved in benzene, and passed through alumina. The coloured part was adsorbed as a double zone (reddish, followed by yellow), as occurs with the mixture of 5 : 8- and 5 : 10-benzpyrene quinones. After elution and further purification by a second chromatography, which entirely removed fluorescent impurities, the coloured product was submitted to reductive methylation. This yielded an intensely fluorescent product, exhibiting the familiar band system of the mixture of 5 : 8- and 5 : 10-dimethoxy-3 : 4-benzpyrenes (Berenblum, Crowfoot,

* The authors estimated that to obtain 1 g. of impure benzpyrene from shale oil by chromatography would entail the use of about 1000 kg. of alumina and many thousands of litres of solvents.

Holiday and Schoental, 1943), thus confirming the presence of benzpyrene in the original fraction.

Quantitative Estimation of 3 : 4-Benzpyrene in Blue Shale Oil.

An aliquot part of the 250 ml. of the benzpyrene-containing fractions of blue shale oil (fractions 68–85, see above) was tested in quantitative dilutions for fluorescence spectra, and the intensities of the bands were compared with those of standard solutions of benzpyrene photographed on the same plate. (For method, see Berenblum and Schoental, 1942.) Attention may be drawn here to the fact that while the quenching effect of O_2 on fluorescence intensity (Bowen and Norton, 1939; Bowen and Williams, 1939; Weil-Malherbe and Weiss, 1942) can influence the sensitivity of the method, the degree of accuracy is not seriously impaired when both the test solutions and the standards are in equilibrium with air. By this means the benzpyrene content of the original crude blue shale oil was found to be of the order of 0·01 per cent. For comparison a similar estimation was made of the benzpyrene content of a sample of carcinogenic coal-tar. This was found to be of the order of 1·5 per cent., i.e. more than 100 times greater than that of shale oil. (This figure of 1·5 per cent. for coal-tar is considerably higher than those quoted by other investigators, such as 0·005 per cent. by Winterstein and Schön, 1934. The latter figure seemed to have been reached by isolation of the compound, and would, for that reason, be very low.) The whole procedure, including the liquid chromatogram, fluorescence spectrography of all the collected samples, and the quantitative estimation, was repeated on another 10 ml. of shale oil. The results obtained were substantially the same.

Fluorescence Spectra of Chromatographic Fractions of Extracts from Solid Shale.

A sample of solid " oil shale " from which shale oil is normally obtained by retorting was finely ground and continuously extracted with chloroform for about a week. The extract, freed from chloroform (a dark brown material of the consistency of soft wax, representing about 1 per cent. of the powdered shale), was dissolved in light petroleum, and fractionated by the method of the liquid chromatogram, as described above, and the separate fractions were examined by fluorescence spectrography.

A large part remained strongly adsorbed on the column (a much higher proportion than was the case with shale oil). Of the small eluable part, only a few of the early fractions showed a simple band system, most of the fractions exhibiting a diffuse fluorescence (Fig. 1). In none of the fractions was there any evidence of the presence of benzpyrene bands. For purposes of confirmation the liquid chromatogram was repeated several times on fresh samples of shale extract, and all the collected samples were examined by fluorescence spectrography. As before, no evidence of benzpyrene bands could be detected in any of the fractions.

DISCUSSION.

The results described above include : (1) The detection of 3 : 4-benzpyrene in blue shale oil ; (2) the induction of tumours in mice with a fraction of the oil entirely free from benzpyrene ; and (3) the failure to detect benzpyrene in extracts of oil shale.

The concentration of benzpyrene in the shale oil (0·01 per cent.) is very low, both by comparison with that found in coal-tar (1·5 per cent.), and when considered in respect of the amount required for pronounced carcinogenic action. Berenblum (1941) has shown that, in a concentration of 0·05 per cent., benzpyrene produced only three skin tumours out of 102 surviving mice after 20 weeks' application. With the blue shale oil (estimated to contain only about 1/5 of that concentration of benz-

pyrene), the tumour yield was 6 out of 10 surviving mice after 18 weeks' application. Hence the benzpyrene present in the oil could not account for more than a very small part of the carcinogenic action. This was confirmed when it was shown that the most potent fraction, obtained from the oil by chromatography, was entirely free from benzpyrene. In this respect, however, blue shale oil does not differ fundamentally from coal-tar, from which benzpyrene-free fractions could be obtained by chromatography which were as strongly, if not more strongly, carcinogenic than a saturated solution in benzene of benzpyrene itself (*B.E.C.C. Rep.*, 1943).

The presence of benzpyrene in blue shale oil, the appearance, in some of the chromatographic fractions, of banded fluorescence of a type not unlike those present in coal-tar, and the general impression gained that, during the chromatographic development, the sequence of appearance of these band systems was similar to that occurring during chromatography of coal-tar, all point to the likelihood that the carcinogenic constituents of blue shale oil belong to the same class of compounds as those in coal-tar. This conclusion seems important in two respects :

(1) The experience both of previous investigators (Twort and Ing, 1928 ; etc.), and of the present authors, indicate that shale oil offers great practical difficulties for the isolation and identification of the various carcinogenic constituents. If the carcinogenic constituents in shale oil belong to the same class of substances as in tar, it seems more reasonable to devote future efforts to their isolation from coal-tar.

(2) In the case of coal-tar it is known that the carcinogenic constituents are not present in the coal from which the tar is obtained, but that they are the products of destructive distillation. Kennaway (1924) made a special study of this problem, and was able to demonstrate a close relationship between the temperature of carbonization of the coal and the carcinogenicity of the tar produced. Since, in the production of shale oil from shale, high temperatures are reached (Kennaway, 1924 ; Smith, 1938), it seemed not unlikely that the carcinogenicity of shale oil might also be due, in part, if not entirely, to the effects of destructive distillation.

The demonstration of benzpyrene in shale oil and its absence in extracts from the original shale give strong support to such a view. This does not necessarily mean that the original shale is void also of the other carcinogens that are present in shale oil in addition to benzpyrene. Nor does it imply that polycyclic hydrocarbons in general—the class of compounds so readily produced by pyrolysis—cannot also occur in nature. The literature appears to have no reference to carcinogenicity tests on unheated extracts from shale. Such tests are now in progress by the present authors.

SUMMARY.

A chromatographic fractionation of blue shale oil yielded fractions that exhibited distinct banded fluorescence spectra, among which the characteristic band system of 3 : 4-benzpyrene was readily detected. Its presence was confirmed by conversion into its dimethoxy derivatives.

The concentration of 3 : 4-benzpyrene in blue shale oil was estimated by spectrofluorimetry to be of the order of 0·01 per cent.

Evidence of the existence of other carcinogenic constituents was demonstrated by the strong carcinogenic activity of a fraction giving no fluorimetric evidence of the presence of benzpyrene.

Benzpyrene was not found to be present in chloroform extracts of the solid shale, from which shale oil is derived by retorting.

The results obtained support the view that the carcinogenic constituents of blue

shale oil belong to the same class of compounds as those responsible for carcinogenic action in coal-tar, and that their presence may be accounted for by temperature effects during the process of its production from the shale.

We are indebted to Scottish Oils Ltd. for generous supplies of blue shale oil and of oil shale. We wish to thank Mr. H. W. Wheal for valuable technical assistance.

REFERENCES.

BERENBLUM, I.—(1941) *Cancer Res.*, **1**, 44.
Idem AND SCHOENTAL, R.—(1942) *Biochem. J.*, **36**, 86.
Idem, CROWFOOT, D., HOLIDAY, E. R., AND SCHOENTAL, R.—(1943) *Cancer Res.*, **3**, 151.
BOWEN, E. J., AND NORTON, A.—(1939) *Trans. Faraday Soc.*, **35**, 44.
Idem AND WILLIAMS, A. H.—(1939) *Ibid.*, **35**, 765.
Brit. Emp. Cancer Camp., Ann. Rep.—(1940), **17**, 202.—(1941) **18**, 163.—(1942) **19**, 58.—(1943) **20**, 53.
COOK, J. W., HEWETT, C. L., AND HIEGER, I.—(1933) *J. chem. Soc.*, 395.
HIEGER, I.—(1930) *Biochem. J.*, **24**, 505.—(1937) *Amer. J. Cancer*, **29**, 705.
KENNAWAY, E. L.—(1924) *J. indust. Hyg.*, **5**, 462.
LEITCH, A.—(1922) *Brit. med. J.*, ii, 1104.
MAYNEORD, W. V., AND ROE, E.—(1936) *Biochem. J.*, **30**, 707.
MILLER, J. A., AND BAUMANN, C. A.—(1943) *Cancer Res.*, **3**, 217.
SANNIÉ, C.—(1936) *Biochem. J.*, **30**, 704.
SMITH, G. H.—(1938) "Scottish Shale Oil." *The Science of Petroleum*, **4**. Oxf. Univ. Press.
TWORT, C. C., AND ING, H. R.—(1928) *Zeitschr. Krebsforsch.*, **27**, 308.
Idem AND FULTON, J. D.—(1929) *J. Path. Bact.*, **32**, 149.—(1932) *Zeitschr. Krebsforsch.*, **35**, 543.
Idem AND LYTH, R.—(1933) *J. Hyg.*, **33**, 464.
TWORT, J. M., AND LYTH, R.—(1939) *Ibid.*, **39**, 161.
WEIL-MALHERBE, H., AND WEISS, J.—(1942) *Nature*, **149**, 471.
WINTERSTEIN, A., AND SCHON, K.—(1934) *Naturwiss.*, **22**, 237.

ULTRAMICROSCOPICAL OBSERVATIONS ON THE MORPHOLOGY AND DEVELOPMENT OF VACCINIA VIRUS *IN VITRO*.

K. B. E. MERLING.*

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London.

Received for publication October 22, 1943.

THE morphological appearance of living vaccinia virus in corneal cells of the rabbit was described by the author (1940*a*) dealing with a period of observation of up to 9 days. In a following paper (1940*b*) the period of observation was extended to 6 months. The life-cycle of vaccinia virus was confirmed, and the survival of the virus *in vitro* was described in its various forms.

TECHNIQUE.

The technique employed in the following experiments was, for the most part, identical with that described previously (1940*a* and *b*). The eyes of the rabbit were inoculated in the usual manner by scarification with the same strain of vaccinia virus kindly made available by Prof. J. McIntosh. After the vaccinal keratitis had well developed, which took about three to six days, thin tangential sections were cut with a sharp knife ; while from the deeper layers scrapings were obtained with the blade of the knife. Similar control preparations were made from uninoculated corneae. The material obtained was placed in a drop of tyrode solution on a thin slide, covered with a coverslip, and gentle pressure applied to expel excess of fluid. The coverslip was then sealed with paraffin wax to prevent evaporation. Observations were carried out either at room temperature or on the warm stage of the microscope at 37° C. To obtain a clear view and avoid overcrowding of the field special care was taken to have only one or two small fragments of corneal tissue on any one slide. In this way up to 20 specimens could be prepared from one cornea. To prevent contamination during the manipulation the best means was found in speed of work apart from the usual maintenance of sterile conditions.

Optical systems giving a high degree of resolution at the highest magnifications with dark-ground illumination were essential.

RESULTS.

Observations on Colonies Derived from Epithelial Cells.

It is a well known fact that cells that are dying undergo a series of changes which appear as various forms of disintegration. In one way or another the liquid or liquefied contents of the cell are detached from the " skeleton " comprising the membrane, the nucleus and other more solid or more solidly attached cell constituents.

Fig. 1 shows droplet disintegration of normal corneal cells ; the droplets are being formed rapidly in sizes varying in diameter from 1μ to 30μ ; they may be spherical

* Sir Halley Stewart Research Fellow.

and detached, as is most frequently the case, or elongated and connected with the cell skeleton ; they may be optically empty, which means that their inside space appears black and thus darker than the surrounding liquid, or they may contain mitochondria of various sizes or other particles, like droplets of fat or liquids, pigment and other granules that could not be identified. Fatty substances always show high refractility; pigments are coloured. Mitochondria appear in various shapes of very low refractility ; they are very numerous if they are seen in fresh specimens. Mitochondria are nearly always in rapid movement, and in the photograph this gives the grey colour to the inside of the droplets and extrusions.

The same sort of droplet disintegration of corneal cells takes place if the specimen was prepared from an animal previously inoculated with vaccinia virus as described above.

Fig. 2 shows a field that is well comparable to Fig. 1. The fundamental appearance is the same but for the presence of the vaccinia virus. It can justly be said that any difference in appearance of the two figures is due to the virus present in Fig. 2. Elementary bodies surround droplets and extrusions, being attached to their surfaces ; in the right-hand upper corner of fig. 2 can be found one large and several small cysts containing elementary bodies in rapid (Brownian) movement. In the visual image the movement and brightness in and of the cysts make them unmistakable ; in the photograph only the brightness is recorded, while the movement of the elementary bodies within the cyst is evened out, and appears as a light haze which is more or less sharply limited by the cyst membrane. The term " cyst " is used to signify a cell extrusion—formed by droplet disintegration—that has separated from the cell, and leads a detached existence floating in the medium or fixed to a glass surface. A further meaning of " cyst " is that it contains a varying number of virus bodies.

The cysts increase in size, some quickly over a few hours, others take weeks, until they finally burst and collapse, at the same time releasing their contents of virus bodies. Occasionally the virus bodies seem to leave the cyst less suddenly, the cyst remaining at its largest extension for a while ; in that case individual virus bodies can be observed inside the cyst or attached to its inner surface. Several such cysts, their contents of virus bodies largely diminished, are shown in Fig. 3.

In Fig. 4 the same field is shown 24 hours later. The large round cyst in the lower left-hand corner of Fig. 3 has collapsed, its membrane is seen crumpled up, its contents of virus bodies have nearly disappeared. In the central cyst (Fig. 3) the virus bodies were less mobile, and are consequently sharper outlined in the photograph. Some of the virus bodies can be seen lying in chains, particularly in Fig. 4, after the collapse of the cyst. Following the collapse of the cyst the virus bodies are not always dispersed freely immediately in the surrounding liquid. In Fig. 4 below the central collapsed cyst there are four white round patches representing daughter-cysts tightly packed with rapidly moving virus bodies.

In Fig. 5 is shown a large extrusion which has burst before it became detached from its cell. The three forms of the virus that represent its life-cycle can be seen as they are being discharged. Elementary bodies, discs and spheres as the life-cycle forms proper and some small secondary or daughter-cysts are present. Discs are most difficult to observe and photograph owing to their very short smaller diameter (short depth). Their presence in a photograph is indicated, for example, in fig. 6, which shows a number of chains of elementary bodies.

In some of the chains occurs a gap about the size of an elementary body ; that gap is occupied by a disc which has not been recorded owing to its low brightness. No connecting filaments (Paschen, 1906, Prowazek, 1907) have so far been seen or recorded between the members of a chain, although it must be assumed that such

links exist, otherwise it would be difficult to account for the appearance of chains. The attempt to make visible connections between the elementary bodies resulted in an image resembling those rendered by the electron microscope (Green, Anderson and Smadel, 1942), which shows the elementary bodies as oblongs with a certain depth (bricks).

Fig. 7 shows the elementary bodies at a magnification of 2900 times and reveals their oblong shape. The resolution achieved in this photograph is most remarkable, because it is beyond the predicted possibilities of the Abbe and other image formation theories of the microscope. The raising of the limit of resolution, and consequently of magnification, was realized by reducing the intensity of the light source by means of polarized discs to such a degree as to produce coincidence between the minimum of photographic registration and the physiological thresholds of perception and differentiation of two light intensities.

In Fig. 8 the white masses represent remnants of cells that are filled with great numbers of virus bodies. The vaguely recognized cell debris serves as a matrix—nutritious and mechanical—for the slowly growing virus colonies. The colonies are mostly in the form of cysts, sometimes connected to some fragment of a cell; in other cases their development from Guarnieri bodies (intracellular colonies) into detached cysts had been followed.

In Fig. 9 low-power view reveals the shape of three epithelial cells. Not much of the cells' substance is left; the space is occupied by virus which, in its arrangement and distribution, indicates where the cells had been. In all such specimens the virus was in rapid movement, and higher magnifications would only tend to blur the photograph. To secure pictures of these appearances at 1000 diameters a specimen had to be found in which movement had ceased just at this phase of the development. This

---

DESCRIPTIONS OF PLATES

FIG. 1.—Droplet disintegration of normal cells; droplets and extrusions, empty and containing mitochondria. × 1000. 10 minutes after removal from rabbit.

FIG. 2.—Similar to fig. 1. Elementary bodies attached to extrusions; two small cysts containing elementary bodies in fast movement. × 1000. 24 hours after removal from rabbit.

FIG. 3.—Large cysts containing small number of virus bodies. × 1000. 24 hours after removal from rabbit.

FIG. 4.—The same field as fig. 3. 24 hours later, large cysts have burst and discharged most of their contents; a few daughter-cysts are in the field. × 1000.

FIG. 5.—Large cyst discharging contents of virus bodies. × 1000. 1 week after removal from rabbit.

FIG. 6.—Chains of elementary bodies; the position of discs is indicated by links missing in the chain of elementary bodies. × 450. 1 week after removal from rabbit.

FIG. 7.—One chain of elementary bodies. Highest resolution so far obtained with visible light shows elementary bodies as of oblong shape with a faint indication of structure. × 2900. 1 week after removal from rabbit.

FIG. 8.—Remnants of cells, completely disintegrated; no cell membranes or other structures left; virus colonies amidst debris. × 450. 2 weeks after removal from rabbit.

FIG. 9.—Outlines of cells after disintegration is complete; space is occupied by virus colonies. × 450. 2 weeks after removal from rabbit.

FIG. 10.—Similar to fig. 8. × 1000. 2 weeks after removal from rabbit.

FIG. 11.—The virus has formed a lawn on the slide. × 1000. 3 weeks after removal from rabbit.

FIG. 12.—Small encysted colonies are appearing on the lawn of virus. × 450. 4 weeks after removal from rabbit.

FIG. 13.—The cysts are growing and altering their shapes. × 450. 5th week.

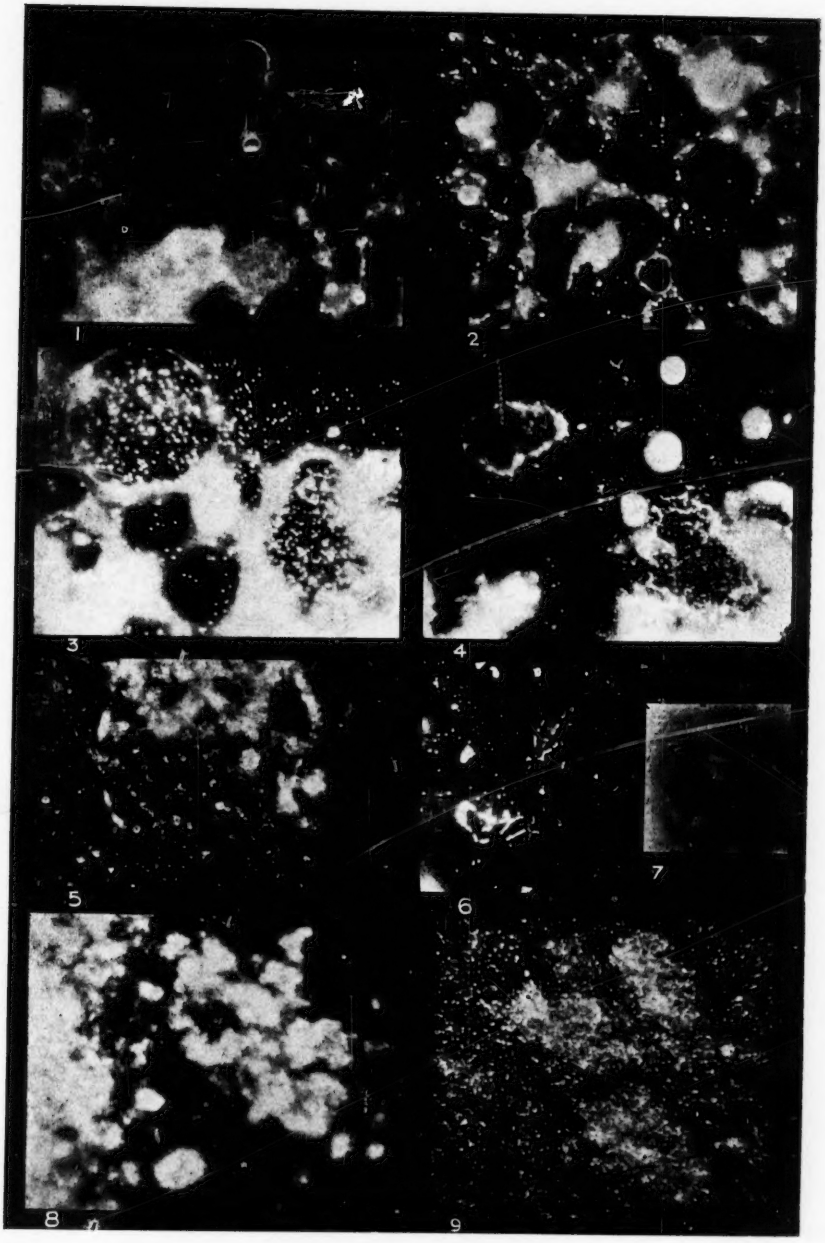
FIG. 14.—The cysts are stretching prior to division. × 450. 5th week. The arrows indicate directions.

FIG. 15.—Cyst after division, moving as a whole. The arrows indicate directions.

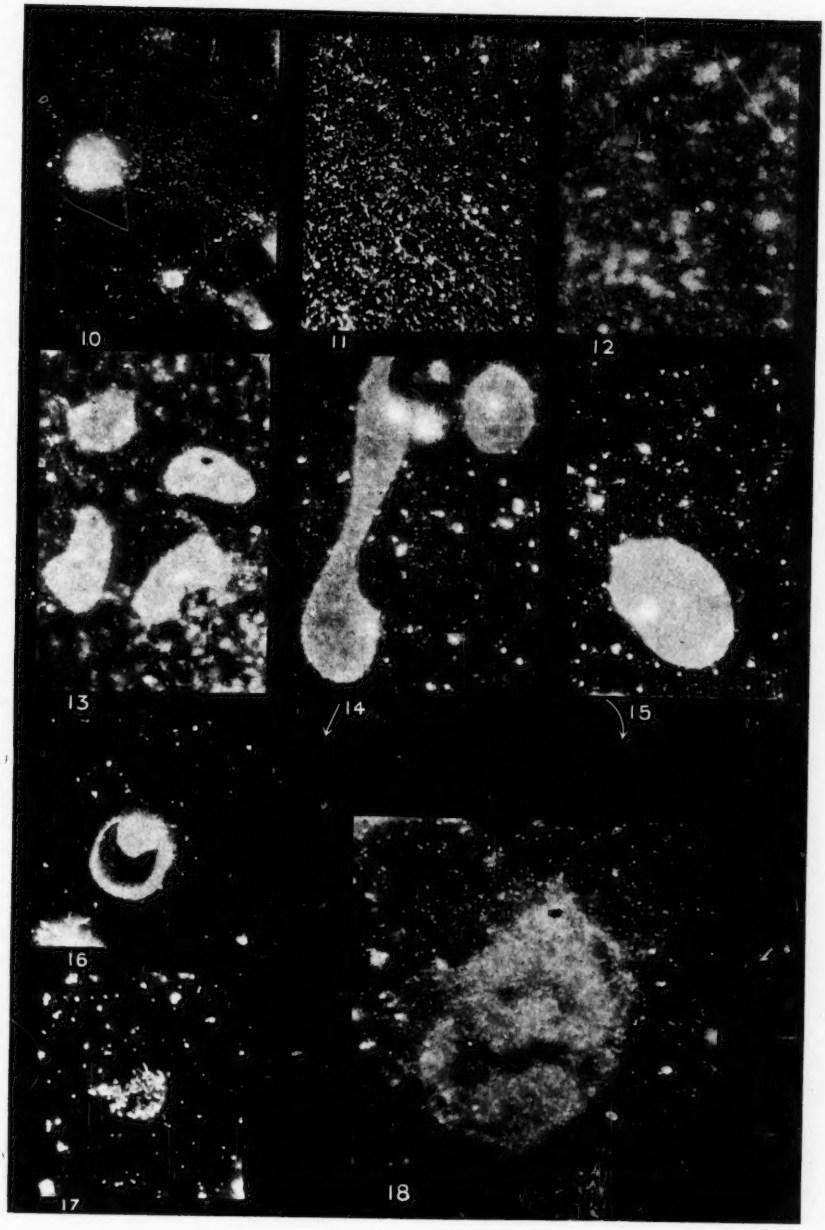
FIG. 16.—A moving cyst is leaving a trail of elementary bodies; inside the cyst a vacuole in the shape of a meniscus surrounds the centre of the colony. × 450. 8th week.

FIG. 17.—Elementary bodies inside a moving cyst. × 1000. 8th week.

FIG. 18.—A moving cyst has come to rest and is breaking up; the virus is being discharged. × 1000. 8th week.



Merling.



Merling.

is easier than it sounds owing to the great number of specimens available. At any rate it is easier than attempting to induce a cessation of activity by outside means. In most cases in which it was tried by lowering the temperature, drawing through of poisons, like various concentrations of prussic acid, the result was a complete upsetting of the arrangement in the specimen, altering its thickness, producing currents, etc.

Fig. 10 shows a photograph of a specimen in which for unknown reasons cells, virus and debris had sunk to the surface of the slide and appeared to adhere to it. In consequence it was possible to take a photograph of this field under fairly good conditions of definition at 1000 diameters. It takes about two to three weeks after removal of the material from the animal for the virus bodies to " consume " their host cells more or less completely. By " more or less" is meant that in one part of the specimen the cells may still be there, though perhaps not intact, whilst in other parts the cells have completely disappeared. If the virus bodies remain mobile, further development within the limits of its life-cycle may take place, or on the other hand, no more obvious changes may occur. A third possibility is the formation of an even layer or lawn by the virus on the slide or coverslip.

Fig. 11 gives an impression of this appearance. There are mostly elementary bodies and spheres ; the elementary bodies are lying singly, in twos, threes, chains of various length and in circles, sometimes around a central individual. Discs are difficult to find owing to their lesser brightness. Spheres stand out owing to their greater brightness. There are a fair number of discs and spheres interspersed with the elementary bodies.

The majority of the virus bodies forming the lawn are immobile. A small number —about 1–2 per cent.—appear to move restrictedly in a circle not exceeding 3μ. They seem to be so confined either by an invisible filament anchored in the centre or by a surface of higher viscosity. This latter explanation seems the more likely, as gelatinization appears to occur fairly frequently within these specimens.

The lawn may remain in this state of apparent dormancy for weeks and indefinitely. But the dormancy may prove to have been spurious or temporary within one more week—if the observations are continued ; gradually the number of virus bodies, moving within one little circle, is increasing until it is realized that small colonies have been formed on the lawn.

Fig. 12 shows a lawn on which about 200 small colonies have been formed. They appear to have a very thin membrane holding their contents together—because now the surrounding medium appears to be again as liquid as it had been before gelatinization, filled with freely moving virus bodies. The small cysts are not round as is the rule with those formed from cell extensions ; there appear a great variety of shapes, whose most impressive characteristic is change. Within another week the cysts grow to large dimensions, measuring sometimes 20 by 30μ across their two diameters. Now a most dramatic and surprising development may take place : the cysts may stretch and divide into two or more.

Fig. 13 shows a number of large cysts of various irregular shapes on a background of small cysts like that in Fig. 12, and the lawn of individual virus bodies. The two lower cysts of Fig. 13 are in the process of division, the cyst in the left corner shaping like an inverted L, the one in the right corner bending in the opposite direction. Following the stretching and dividing of a cyst there becomes apparent a change of place—the cysts as a whole move.

Fig. 14 shows moving cysts ; the direction of the movement is indicated by arrows. In the case of Fig. 14 the movement across the field began actually before the division of the large extremely stretched cyst into four daughter-cysts. The round cyst in fig. 14 is stationary.

The large cyst of Fig. 14 divided into four daughter-cysts, of which three have moved out of the field of view while one is still there, moving very slowly in the direction of the arrow, Fig. 15.

Until this time—the sixth week after the material had been taken from the animal—continuously small cysts had been formed, grown, divided, burst and so on in seemingly endless repetition. During the following two weeks indications appeared that the process was coming to an end. No more new cysts were being formed—on Fig. 15 the background of lawn and small cysts seems to be thinner. Gradually the number of free-moving virus bodies decreased mainly owing to dispersion, but as well to their becoming fixed to one of the glass surfaces.

The movement of the cysts slowed down.

In Fig. 16 a cyst is shown at this stage. There is still a great number of virus bodies moving as lively as ever inside the cyst. But there is not as much space inside the cyst as there had been ; a vacuole in the shape of a meniscus has been formed. The cyst appears to be moving in the direction of some cell debris, spilling virus bodies forming a trail. This cyst did not burst, but disintegrated slowly, some of the virus bodies remaining on the spot, others dispersing.

Fig. 17 shows a small cyst that had stopped its movement for a short while. It was possible to focus on the layer of virus bodies at the base of the cyst, which can be discerned quite plainly. The arrangement of the virus bodies indicates the direction of the movement of the cyst. The membrane of the cyst is not in focus, and appears only as a faint halo around the group of virus.

A comparison of the cysts pictured in Figs. 13–16 with those in the previous publication (1940*b*) on plates L and LII, Figs. 4 and 5 and 14–23, shows the similarity between the two types, indicating that the character of the cysts is the same, although their origin was different. In Fig. 18 a cyst is shown similar to that in Fig. 16, but at a higher magnification. The trail of shed virus bodies is still discernible, though the swarming virus is leaving the now immobile cyst in many directions. The dispersal of the virus has also blurred the outline of the previously clearly defined vacuole which showed meniscus shape while the cyst was in motion.

The ultimate end of the development of the virus in these specimens was uniformly gradual immobilization and slow loss of brightness of the virus bodies. In this stage the coverslip can easily be removed and the specimen stained. The best stain appeared to be Herzberg's Victoria blue. Stained specimens showed the great number of virus bodies and very few cells amidst some cell debris. Control specimens of normal (not inoculated) cornea kept for the same length of time showed cells in varying degrees of disintegration but were, of course, negative as regards virus bodies.

DISCUSSION.

The observations described in the present paper are concerned with further details of colony formation of vaccinia virus under the conditions of the experiments. It was possible with improved photographic technique to obtain photomicrographs of elusive phases in the life-cycle of the virus, like the formation of chains of elementary bodies, of discs and small spheres. Of importance in the way of ultramicroscopical technique appears the magnification and resolution achieved, for instance, in Fig. 7, which surpasses anything comparable, i.e. obtained with visible light. Ultra-violet photomicrographs by Barnard show the elementary bodies of vaccinia as slightly elongated rings. The chain of elementary bodies in Fig. 7 appears more like a row of oblongs, resembling the photographs of the virus taken with the electron microscope at least in outline. It would be asking too much of even the best ultra-microscope working with white light to render visible the internal granules in elementary bodies as they

are imaged by the electron beam. It must, however, be pointed out that the limits of the ordinary microscope have not been reached yet, nor its possibilities exhausted ; in a recent paper by H. H. Hopkins (1943) theoretical reasons were given why resolution should be increased in one particular plane of polarized light. The author has used polarized light to this purpose with good effect (1937), although the theoretical considerations leading to its use were quite different from those put forward by Hopkins.

The chains of elementary bodies which have been recorded in several instances are in no way different from those observed in the case of the virus of pleuropneumonia. This organism is regarded by some authors— for instance, Ledingham and his collaborators— as a fungus belonging to the family of Actinomycetaceae, not a virus, for reasons that need not be discussed here. But it may be pointed out that the opposite opinion regarding that micro-organism as a virus receives some support from the finding of an identical life-cycle for an undoubted virus like vaccinia.

The formation of new colonies after the complete disappearance of host cells and even their broken fragments was one of the surprising results of these observations. It shows that metabolic products present in the liquid are stable and so abundant that they are sufficient for the inception of new colonies, and that the nutritious requirements of the virus, though highly specific, are extremely small. On the other hand, while some metabolic cell-products were obviously of the highest importance for the continued life of the virus *in vitro*, substances freed by the disintegration of the dying cells appeared to fulfil a similar function, as only in this way could a new formation of virus colonies at these late stages be accounted for.

Another surprise was furnished and recorded photographically in the movement of entire virus colonies—i.e. the locomotion, division and subdivision of cysts into two or three equally mobile or resting cysts. These cysts seemed to have an extremely thin membrane, which showed up the virus bodies inside very well, and the appearance of fissures previous to division was very marked and could be recorded.

SUMMARY.

Prolonged observations on microscopical specimens of corneal cells containing vaccinia virus resulted in repeated confirmation of a life-cycle of the virus and the formation of new virus colonies originating from dead cells or cell debris. Further, the formation of new virus colonies occurred in a sheet of virus bodies, though living cells were absent. These colonies in the form of cysts were observed to move on the slide and to divide into daughter-cysts. Within the cysts all the forms of the life-cycle of the virus were found again. With further improved technique photographs were obtained of chains of elementary bodies, discs, etc., using highest magnifications and maximum resolution.

I am indebted to Prof. James McIntosh for his interest and criticism, and to the Council of the Middlesex Hospital Medical School for facilities in carrying out this work.

REFERENCES.

BARNARD, J. E.—(1932) *J. Roy. micr. Soc.*, ser. 3, **52**, 234.

GREEN, R. H., ANDERSON, T. F., AND SMADEL, J. E.—(1942) *J. exp. Med.*, **75**, 651.

HOPKINS, H. H.—(1943) *Proc. phys. Soc.*, **55**, 2, 116.

LEDINGHAM, J. C. G.—(1933) *J. Path. Bact.*, **37**, 393.

MERLING, EISENBERG-, K. B.—(1937) *J. Quekett micr. Club*, 1 ,324.—(1940*a*) *J. Path. Bact.*, **50**, 279.—(1940*b*) *Ibid.*, **50**, 391.

PASCHEN, E.—(1906) *Münch. med. Wschr.*, **1**, 111, 2391.

PROWAZEK, S. VON.—(1907) *Arch. Protistenk.*, **10**, 336.

THE PRODUCTION OF DRUG-RESISTANT CULTURES OF BACTERIA *IN VITRO* AND A STUDY OF THEIR INTER-RELATIONSHIPS.

J. McINTOSH AND F. R. SELBIE.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W. 1.

Received for publication November 1, 1943.

CERTAIN new and interesting facts concerning bacterial drug resistance have come to light in connection with our investigations on bacterial chemotherapy, and it is with these that we propose to deal in this communication.

Soon after the introduction of sulphanilamide as a chemotherapeutic drug, the appearance of resistant strains was first reported in clinical cases with deleterious effect upon the efficiency of the treatment by Maclean, Rogers and Fleming (1939). We therefore set out to investigate how, and with what facility drug-resistance could be produced *in vitro* against the sulphonamides and other chemotherapeutic drugs, and if possible to gain some insight into the mechanism of this change, and how it might be circumvented.

MATERIAL AND METHODS.

Media.

When dealing with sulphanilamide, and particularly with a highly specialized organism such as *Streptococcus pyogenes* (Group A), a careful balance has to be maintained between the nutritive value of the medium and its antibacteriostatic properties. The medium found most satisfactory was 1/5 strength ordinary meat broth containing 1 per cent. bactopeptone, 0·2 per cent. glucose, and varying amounts of horse serum. The actual amount of horse serum was usually about 1 per cent., but had to be adjusted to each particular batch of medium so that it just allowed of visible growth in 24 to 48 hours. This medium did not inhibit the action of sulphanilamide in the concentrations used in the experiments. With the other drugs these precautions so far appear to be unnecessary, and as a rule ordinary broth was found satisfactory.

Organisms studied.

The organisms investigated were several strains of *Streptococcus pyogenes* (Group A) freshly isolated from wounds, and one single strain of *Staphylococcus pyogenes* (B.S.I. 152).

Inocula.

In the sulphonamide experiments great attention had to be paid to the size of the inoculum, and the number of viable bacteria were kept in the neighbourhood of 500 to 1,000 organisms. With staphylococci and against other drugs there was not the same necessity, but in general the inoculum should be kept constant within definite limits if comparable results are to be obtained.

Titration of chemotherapeutic substances.

The dilutions of the drug in the media were varied according to its chemotherapeutic activity. In general with the sulphonamides each step was a 5-fold dilution (Fig. 1), whilst with the other drugs it was a 2-fold serial dilution, as shown in Table I. The cultures were incubated for 18 hours, and the bacteriostatic concentration of the drug was then read as the lowest dilution which inhibited growth of the organism. With streptococci the test period was lengthened to 48 hours.

PRODUCTION OF RESISTANCE.

The method used to produce drug resistance was based on that employed by Levaditi and McIntosh (1910) to produce arsenic- and antibody-resistant strains of trypanosomes, the underlying principle being to train organisms step by step to survive increasing concentrations of the drug. In practice this was done by making successive titrations of the action of the drug against the organism, and at each titration

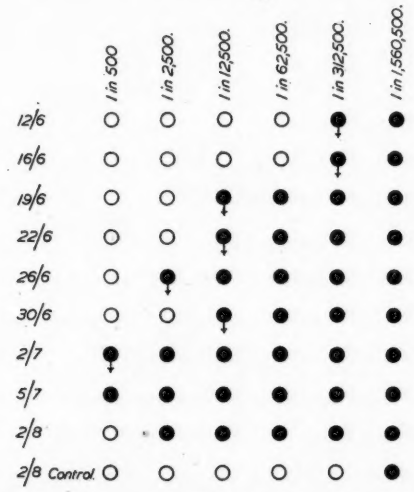


FIG. 1.—Production of a sulphanilamide-resistant culture of *Streptococcus pyogenes*. Each circle represents a test-tube. Blocked out circles represent growth, and the arrows indicate tubes from which the next titration of the action of the drug was made.

taking the bacterial inoculum from the tube with the greatest concentration of the drug showing growth in the previous test. Control inocula were made on solid medium at intervals in order to ensure that no contaminating organism had crept in.

The method is illustrated in Fig. 1, where it will be seen that after 6 serial passages a *Streptococcus pyogenes* was trained to withstand 600 times as much sulphanilamide as at the beginning of the experiment. A final titration made a year later showed that this induced resistance is apparently permanent. Four such strains were produced with much the same facility. With some of the strains of streptococci, however, there was a tendency to develop a certain degree of spontaneous resistance on repeated subculture on the basic medium.

Using the same technique with plain meat broth four cultures resistant to four different drugs were prepared from a single strain of *Staphylococcus pyogenes*. These staphylococcus cultures were able to withstand a 64-fold increase in propamidine and

TABLE I.—*Production of Drug Resistance in* Staphylococcus pyogenes.

Successive subcultures.	Highest concentration of drug permitting growth.			
	Propamidine.	Proflavine.	Quindoline methochloride.	Penicillin (Standard solution =200 units per c.c.)
1	1/80,000	1/160,000	1/80,000	1/20,000
2	1/40,000	1/80,000	1/40,000	1/10,000
3	1/20,000	,,	1/20,000	,,
4	1/10,000	1/40,000	1/10,000	1/5,000
5	,,	,,	,,	1/2,500
6	,,	1/20,000	,,	,,
7–8	,,	,,	,,	,,
9	,,	,,	,,	1/1,250
10	1/5,000	1/10,000	,,	,,
11	,,	,,	,,	1/625
12–13	,,	,,	,,	,,
14	1/2,500	,,	,,	1/312
15–20	,,	,,		
21	1/1,250	,,		

penicillin, a 16-fold increase in proflavine, and an 8-fold increase in quindoline methochloride respectively. These experiments are shown in detail in Table I, in which there is also evidence that the response of the micro-organism to training differs from drug to drug. Thus resistance to propamidine and penicillin was induced rapidly at first, and then increased gradually by steps at intervals of from 2 to 7 passages, whereas with proflavine and quindoline methochloride no further increase in resistance was obtained after the 10th and 4th passages respectively.

These experiments suffice to show that with a suitable organism and some drugs the phenomenon of drug resistance may be elicited with facility in the test-tube. On the other hand in the case of streptococci, resistance was produced only with difficulty to a low figure with the acridine drugs and to a moderate extent with propamidine (10-fold). There was also some difficulty in producing resistant cultures of the staphylococcus to some drugs, such as 2 : 7-diaminoacridine, 5-aminoacridine, rivanol, proflavine and crystal violet.

CROSS-RESISTANCE.

The next step in the investigation was to find to what extent a culture resistant to one drug might still be susceptible to other drugs of similar and dissimilar chemical constitution. Titrations were therefore made of the action of various drugs against these drug-resistant cultures. Sulphanilamide-resistant strains showed some degree of resistance to a number of other sulphonamides, with the notable exception of marfanil.

In the case of *Staphylococcus pyogenes*-resistant cultures, some interesting new facts came to light at an early stage of the work. As is shown in Fig. 2, there was no cross-resistance between sulphathiazole or penicillin and the other drugs tested, as has been shown for penicillin and sulphathiazole by Rammelkamp and Maxon (1942). On the other hand, the propamidine-resistant culture was resistant to proflavine and the proflavine-resistant culture was also resistant to propamidine. It was therefore decided to produce resistant cultures to other acridines in order to find out whether this phenomenon of cross-resistance was general in the acridine series and related

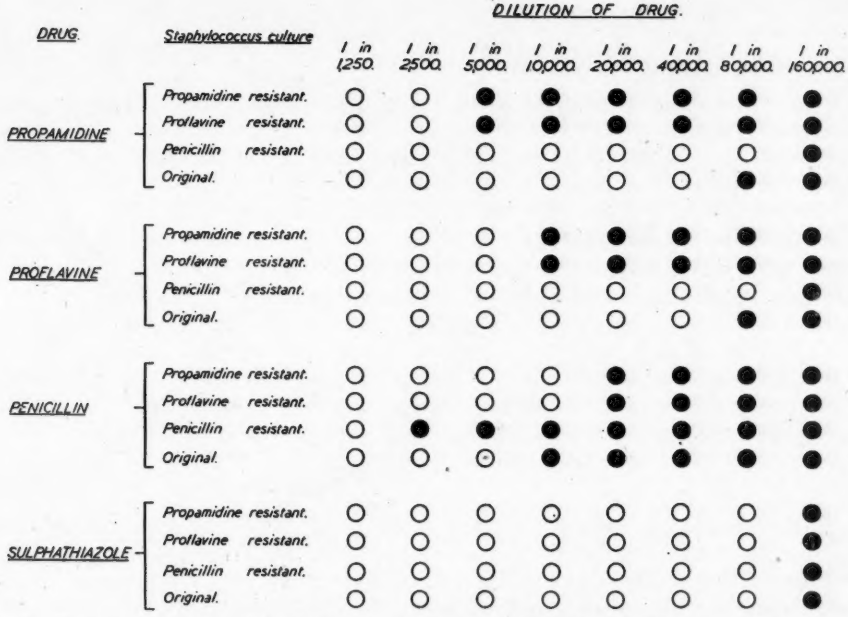


FIG. 2.—Bacteriostatic action of proflavine, propamidine, penicillin and sulphathiazole on drug-resistant and unmodified cultures of a strain of *Staphylococcus pyogenes*.

TABLE II.—*Cross-Resistance of Drug Habituated Cultures of a Strain of* Staphylococcus pyogenes.

Expt.	Culture habituated to.	Bacteriostatic concentration of drug expressed as multiple of that required against unmodified culture.					
		Propamidine.	Proflavine.	Quindoline methochloride.	2:7-diamino-acridine.	5-amino-acridine.	Rivanol.
1.	Propamidine . .	64	4	4	1	2	1
	Proflavine . . .	64	8	8	1	2	1
	Quindoline methochloride . .	32	4	4	1	1	1
2.	Propamidine . .	16	8	4	½	1	2
	Proflavine . . .	32	8	8	1	2	2
	Quindoline methochloride . .	2	4	8	1	4	2
	2 : 7-diaminoacridine .	16	8	4	2	2	1
	5-aminoacridine . .	2	2	1	1	2	2

compounds. Two experiments of this type were done and the results are shown in Table II.

In Experiment 1 titrations of the actions of a number of drugs were made against three of the resistant cultures already described and shown in Table I. For Experiment 2 a second series of cultures was habituated for 12 successive titrations to the

same drugs and also to 2 : 7-diaminoacridine (Albert, Francis, Garrod, and Linnell, 1938), and 5-aminoacridine (Rubbo, Albert, and Maxwell, 1942). In the table the concentrations required for bacteriostasis are expressed as multiples of the inhibiting dose for the unmodified culture. Since the titrations were done by serial 2-fold dilutions only a 4-fold dilution can be considered as being significant of increased resistance.

It will be seen that the cross-resistance between propamidine and proflavine already shown in Fig. 2 is confirmed. Cross-resistance between propamidine and quindoline methochloride is also shown in Experiment 1, and to a much less extent in Experiment 2. To the two additional drugs 2 : 7-diaminoacridine and 5-aminoacridine no increase of resistance was obtained, but the culture habituated to 2 : 7-diamino-

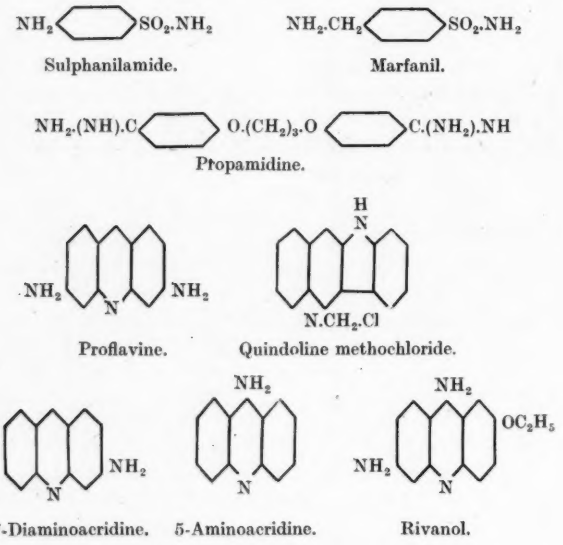


FIG. 3.—Chemical formulae of bacteriostatic drugs mentioned in the text.

acridine showed considerable resistance to the action of propamidine and proflavine. One remarkable feature is the much higher degree of resistance obtained to propamidine, not only by habituation to propamidine, but under the stimulus of other drugs. Another interesting point is the difference in cross-resistance obtained by habituation to quindoline methochloride in the two experiments. In the first experiment the culture became 8 times more resistant to propamidine than to quindoline methochloride, whereas in the second experiment no significant degree of resistance to propamidine but some resistance to 5-aminoacridine was obtained. General conclusions which may be drawn from these experiments are :

(i) In these experiments a much higher resistance to propamidine was obtained than to any of the other drugs, even when the cultures were exposed to other drugs.

(ii) Drugs to which resistance may not be easily produced (2 : 7-diaminoacridine and 5-aminoacridine) tend to be fully active against cultures made resistant to closely related compounds such as proflavine and quindoline methochloride.

(iii) Another peculiarity would appear to be that cultures can respond to training to the same drug in different ways as with quindoline methochloride.

Double resistance.

In addition to cross-resistance another feature we have come across is double resistance, namely that a culture made resistant to a particular drug can also be made resistant to a second drug by treating the resistant culture to gradually increasing doses of the second drug ; this has been done with proflavine and penicillin and also with propamidine and penicillin. This phenomenon does not appear to bear any relation to cross-resistance.

DISCUSSION.

By the use of the technique described we have shown with what facility bacteria can be made resistant *in vitro* to chemotherapeutic substances. This process does not appear to be one of selection from resistant organisms present in the original culture, because these would be expected to grow from the beginning even in the higher concentrations. The mechanism of this resistance appears therefore to be a training of a bacterium to alter its metabolism in such a way that it becomes less susceptible to the chemotherapeutic substance. Resistance therefore must be related to the mode of action of the drug, and this in fact has been shown in the case of certain strains of pneumococci and staphylococci where the resistance is related to the production of excess of para-aminobenzoic acid (Macleod, 1940 ; Landy, Larkum, Oswald, and Streightoff, 1943). This, however, does not appear to be true of all sulphonamide-resistant organisms (Tillett, Cambier, and Harris, 1943), a fact which we have also noted.

Equally significant has been the demonstration that resistant cultures of bacteria may show a high resistance to other drugs often of a completely dissimilar chemical composition, and that at times they may show little or no resistance to similar drugs. Thus it may be said that similar drugs may act in dissimilar ways, and again that dissimilar drugs may act in similar ways. These findings would therefore appear to support the view that the substituent groups are the important elements in chemotherapeutic drugs.

It appears evident that with these resistance experiments, cross-resistance relationships can be determined which can give a clue to the mechanism of the mode of action of the drug and even provide a means of classifying drug action, even though the blocking mechanism is imperfectly known.

Lastly the facility with which resistance strains can be produced is of great importance in the practical application of chemotherapy, and indicates strongly the necessity for the use of maximal doses to produce an immediate result. Truer than ever is Ehrlich's motto " Therapia sterilisans magna " in chemotherapy.

SUMMARY.

Drug-resistant cultures of bacteria can, with great ease, be produced *in vitro* against some chemotherapeutic drugs, and with considerable difficulty against others.

The acridine series of drugs falls into two groups. To those in the first group, resistant cultures could be easily produced ; in the second group, resistance was obtained only with difficulty or not at all. Resistant cultures to the first group showed distinct cross-resistance between themselves, but none of these were resistant to any members of the second group.

Cultures resistant to propamidine were obtained with great facility, not only by exposure to the action of propamidine, but also by the action of some acridine compounds, and were found to be resistant to the members of the first acridine group.

In the case of sulphonamides, cultures resistant to sulphanilamide were resistant or partially resistant to the other members of this group with the exception of marfanil. Resistance once acquired seems to be permanent, and so far we have not been successful in reducing it *in vitro*.

REFERENCES.

ALBERT, A., FRANCIS, A. E., GARROD, L. P., AND LINNELL, W. H.—(1938) *Brit. J. exp. Path.*, **19**, 41.
LANDY, M., LARKUM, N. W., OSWALD, E. J., AND STREIGHTOFF, F.—(1943) *Science*, **97**, 265.
LEVADITI, C., AND MCINTOSH, J.—(1910) *Bull. Soc. Path. exot.*, **3**, 368.
MACLEAN, I. H., ROGERS, K. B., AND FLEMING, A.—(1939) *Lancet*, i, 562.
MACLEOD, C. M.—(1940) *J. exp. Med.*, **72**, 217.
RAMMELKAMP, C. H., AND MAXON, T.—(1942) *Proc. Soc. exp. Biol., N.Y.*, **51**, 386.
RUBBO, S. D., ALBERT, A., AND MAXWELL, M.—(1942) *Brit. J. exp. Path.*, **23**, 69.
TILLETT, W. S., CAMBIER, M. J., AND HARRIS, W. H.—(1943) *J. clin. Invest.*, **22**, 249.

THE SENSITIZING AND INDICATOR ACTION OF VICTORIA BLUE AND JANUS GREEN ON THE FLOCCULATION REACTION FOR SYPHILIS.

F. M. BERGER.

From the Public Health Laboratory, County Hall, Wakefield.

Received for publication November 9, 1943.

DEAN (1937) found that isamine blue could act as an indicator of the reaction between an antigen and its homologous antibody. The addition of the dye to a mixture of horse serum and dilute antiserum produced a precipitate which was easily visible because it took up all the dye from the supernatant fluid. Prof. P. L. Sutherland suggested the possibility of using isamine blue as indicator in serological tests for syphilis. This dye did not prove entirely satisfactory, but certain other dyes were found which, when used together with suitable alcoholic heart extracts, caused in syphilitic sera not only the formation of coloured precipitates, taking up all the dye from the solution, but also very markedly increased the sensitivity of the reaction.

The Effect of Various Dyes on the Flocculation Test for Syphilis.

When investigating the action of various dyes on mixtures of alcoholic heart extracts with syphilitic sera, the following simple technique was used : To 10 volumes of Kahn's antigen, containing 0·6 per cent. of cholesterol, one volume of tinct. benz. co. B.P. was added. Of this stock antigen 0·2 ml. were pipetted to the bottom of a serological tube and a small loopful of powdered dye added. In a few seconds, after the dye had gone into solution, 2 ml. of 0·9 per cent. sodium chloride solution was blown rapidly into the antigen-dye mixture from a pipette.

Suspensions obtained in this way were tested with syphilitic and normal sera immediately after preparation. Sera were heated to 56° C. for 30 minutes before testing. One drop of the antigen suspension was mixed on a slide with an equal

amount of serum. The slide was rocked for 4 minutes, and the fluid examined for the presence or absence of floccules with the unaided eye. Suspensions containing no dye did not show flocculation in syphilitic or normal sera.

Suspensions containing varying but known amounts of dye were also prepared and tested with syphilitic sera of varying strength. In this way the relative activity of various dyes was studied.

The behaviour of many dyes was also investigated by a "tube test." The antigen suspension containing a suitable dye was prepared exactly as for the slide test. Of this suspension 0·1 ml. was pipetted into a small serological tube and 0·1 ml. of serum added. The mixture was shaken in a Kahn shaking machine for 3 minutes and 0·5 ml. of saline solution was added. The results of the test were then read at once. With normal sera clear, transparent fluids were obtained. Syphilitic sera in the absence of dyes usually caused some flocculation, which manifested itself by the opalescent or turbid appearance of the fluid. In the presence of certain dyes this flocculation was intensified, causing the appearance of large floccules easily visible with the naked eye, and the dyes were adsorbed by the precipitate.

In all 78 different dyes were examined. According to their behaviour in the tests just described the dyes could be divided into three groups. The first group comprised all those dyes which did not have any influence on the reaction. The intensity of

TABLE I.—*Classification of Certain Dyes According to their Action on the Flocculation Reaction for Syphilis.*

Group I. No effect.	Group II. Slight sensitization. No indicator action.	Group III. Marked sensitization. Indicator effect.
Alizarin.	Acriflavine.	Janus green B.
Anilin blue w.s.	Brilliant cresyl blue.	Victoria blue 4R.
Azolitmin.	Brilliant green.	Anilin blue spirit sol.
Benzopurpurin.	Chrysoidine.	Isamine blue.
Congo red.	Crystal violet.	Janus black.
Eosin water sol.	Dahlia.	Methyl green.
Erythrosin.	Eosin alc. sol.	Nile blue sulphate.
Fuchsin, acid and basic.	Gentian violet.	Pyronine G.
Haematoxylin.	Janus red.	Sudan black.
Indigocarmine.	Malachite green.	
Methyl red.	Methyl blue.	
Naphthol green B.	Methyl violet.	
Nigrosin.	Methylene blue.	
Orange G and II.	Neutral red.	
Orcin.	Phenosafranin.	
Resazurine.	Prussian blue.	
Scarlet R.	Resorcin-fuchsin.	
Sudan III.	Safranin.	
Sulphophthaleins (various).	Victoria blue B.	
Trypan blue.	Toluidine blue O	
Trypan red.		

the reaction was not increased in the presence of these dyes, and the dyes, when at all soluble in water, were not taken up by the precipitate. Sudan III, fuchsin, benzopurpurin and many other dyes belonged to this group. Dyes of the second group

sensitized the reaction. In their presence smaller amounts of syphilitic antibody sufficed to cause flocculation than in their absence. The sensitizing effect was not, however, very powerful, and relatively large amounts of dye had to be present for the effect to take place. Although the floccules which formed in syphilitic sera were coloured, the greater part of the dye remained in solution. Methylene blue, neutral red, crystal violet, acriflavine and many others belonged to this group, which can be briefly described as having a weak sensitizing and no indicator effect.

The third group of dyes comprised those having both marked sensitizing and indicator effects. In the presence of these dyes heavy flocculation occurred rapidly, even with sera containing only little syphilitic antibody. The precipitate was intensely coloured and took up all the dye from the solution, leaving a water-clear supernatant fluid from which the whole of the dye disappeared. This was in striking contrast to the tubes containing normal sera, which showed a uniformly coloured clear fluid. Janus green and Victoria blue appeared to be the best sensitizers. A few other dyes mentioned in Table I had a similar but not quite so marked effect.

Isamine blue is only slightly soluble in alcohol, and due to this fact its indicator and sensitizing effects were bound to be weak. When, however, finely powdered isamine blue was suspended in the alcoholic heart extract, after dispersion a good indicator and sensitizing effect could be observed. These effects could not be obtained when similar amounts of dye were dissolved in the saline solution used for dispersion of the alcoholic extract and not in the extract itself.

The Action of Janus Green and Victoria Blue.

To exert their indicator and sensitizing effects these dyes had to be dissolved in the alcoholic heart extract. As in the case of isamine blue, dissolving of the dyes in the saline solution used for dispersion of the alcoholic extract did not cause any or only little sensitization. Addition of the dyes to mixtures of antigen with antibody did not cause sensitization of the reaction. In the absence of tinct. benz. co., Victoria blue and Janus green behaved differently. In the case of Janus green only the indicator effect of the dye was lost and the sensitizing action maintained, whereas in the case of Victoria blue both effects were lost.

The relation between the amount of dye present in the antigen and the sensitivity of the test was also investigated. Fig. 1 shows that the sensitivity of the reaction increases with the amount of dye. It shows, further, that in the presence of relatively large amounts of dye the slide test was more sensitive than the tube test, whereas in the presence of small amounts of dye the reverse was true.

The existence of the sensitizing action of Victoria blue and Janus green could be demonstrated in three different ways. Firstly, when alcoholic heart extracts were dispersed in excessive amounts of saline solution, so that no reaction occurred in the presence of syphilitic sera, nevertheless powerful precipitation was observed in the presence of these dyes. Secondly, syphilitic sera containing amounts of syphilitic antibody too small to react with a given antigen without dye showed marked flocculation in the presence of Victoria blue or Janus green. Finally, these dyes sensitized the antigen-antibody complexes to the flocculating action of electrolytes, as in the presence of these dyes smaller amounts of electrolytes were sufficient for precipitation than in their absence.

The fortifying action of Janus green was very much stronger than that of Victoria blue, as Janus green could not only intensify the action of cholesterin, but was also capable of taking over to a considerable extent the fortifying action of cholesterin. (Samples of dyes used in these experiments were examined for the presence of mineral

salts, particularly sodium chloride and sulphate, and these were not found to be present.)

Table II illustrates the effects caused by Victoria blue and Janus green, and compares them with the fortifying action of cholesterin in the presence of two different concentrations of electrolytes.

An ether pre-extracted alcoholic beef heart extract, to 10 parts of which 1 part of tinct. benz. co. B.P. was added, was used as antigen. The requisite quantities of cholesterin or dye or both were dissolved in portions of this antigen, as indicated in Table II. One volume of the various antigens was dispersed in 10 volumes of (*a*) 0·85 per cent. and (*b*) 2 per cent. sodium chloride solutions. These antigen suspensions were tested at once and at various periods of time after preparation with normal sera and with graded dilutions of syphilitic sera.

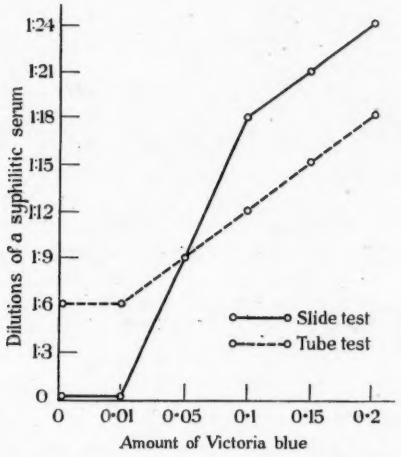


FIG. 1.—The influence of Victoria blue on the sensitivity of the flocculation reaction for syphilis.

The syphilitic serum consisted of a mixture of 10 syphilitic sera and graded dilutions were prepared, using a sample of mixed normal serum as diluting fluid. The same set of dilutions was used throughout the experiment. All tests were carried out on the slide, and the results were read with the naked eye after 4 minutes rocking. The figures in Table II indicate the highest dilution of syphilitic serum giving a definite reaction with the respective antigen suspension. For example, figure 6 means that the respective antigen suspension gave a well-marked flocculation in a 1 in 6 dilution of syphilitic serum. The figure 0 means that no reaction was observed in undiluted syphilitic serum, and 0·5 signifies that only a doubtful reaction was obtained with undiluted syphilitic serum. Normal sera did not react with any of the antigen suspensions at any of the time intervals noted in Table II.

Table II shows that in the absence of both cholesterin and dye the antigen suspension did not show flocculation with syphilitic sera, not even in high concentrations of electrolytes. In the presence of suitable amounts of cholesterin and relatively large amounts of electrolytes a sensitive antigen suspension was obtained, provided that a sufficiently long time interval was allowed for ripening. In this case the sensitivity of the antigen was a function not only of the amount of electrolytes present, but also of the time interval which elapsed after addition. In the absence of cholesterin,

TABLE II.—*The Sensitizing Action of Victoria Blue, Janus Green and Cholesterin in the Presence of 0·85 and 2·0 per cent. Sodium Chloride Solution.*

Antigen.	Cholesterin. %	Dye. %	Sodium chloride content of dispersing fluid. 0.85%. Time interval after dispersion. Hours. 0.	1.	2.30.	5.	8.	24.	48.	2.0%. Time interval after dispersion. Hours. 0.	1.	2.30.	5.	8.	24.	48.
Alcoholic heart extract 10 p., tinct. benz. co. 1 p.	—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0·6	—	0	0	0·5	0·5	0·5	1	1	0·5	1	3	4·5	4·5	6	9
	—	V.B. 0·25	0	0	0	0	0	1	3	0	0	0	0	0	0	0·5
	—	J.G. 0·25	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	0·6	V.B. 0·17	9	9	9	9	9	9	9	15	15	15	15	15	15	15
	0·6	J.G. 0·2	15	15	15	15	15	15	15	15	15	15	15	15	15	15

V.B., Victoria blue ; J.G., Janus green. The figures signify the highest dilution of a sample of syphilitic serum in which flocculation occurred. For further explanation see text.

Victoria blue and Janus green behaved differently. Victoria blue possessed a cholesterin-like action in the presence of low electrolyte concentrations, i.e. it had some fortifying effect, which, however, needed time to make its full appearance. In the presence of 2 per cent. of sodium chloride (when cholesterin was not contained in the antigen) Victoria blue acted as protective colloid and not as sensitizer of the antigen suspension. Janus green, however, both in the presence of low and high sodium chloride concentrations, markedly sensitized the antigen suspension, even in the absence of cholesterin. The remarkable feature of this sensitization was that it took place immediately after dispersion of the antigen, and that it then remained at a constant level for relatively long periods of time. In the presence of cholesterin both Janus green and Victoria blue possessed this peculiar effect of immediate sensitization to a still more marked extent. Under such conditions the sensitizing effect of Victoria blue was increased in the presence of higher electrolyte concentrations, whereas the sensitizing effect of Janus green was of the same order in the presence of both 0·85 per cent. and 2 per cent. sodium chloride concentrations.

The Nature of the Sensitizing Action of Dyes.

The mechanism of the sensitizing action of Janus green and Victoria blue appears to differ from that of cholesterin. Cholesterinized antigens must be allowed to ripen in order to attain maximal sensitivity, whereas antigens sensitized by dyes possess maximal sensitivity immediately after dispersion. The fact that cholesterin intensifies the sensitizing action of dyes and vice versa also suggests different mechanisms of action of the two sensitizers.

Browning and Mackenzie (1924) brought forward evidence to show that the sensitizing action of cholesterin is due to its influence on the physical state of the emulsion and to the operation of a chemical factor. Eagle (1930) believes that the coarser dispersion of the alcoholic antigen which takes place in the presence of cholesterin is the sole cause of its sensitizing effect. He therefore assumed (Eagle, 1931) that other water-insoluble, alcohol-soluble substances would have the same effect. Water-insoluble, alcohol-soluble dyes as Sudan III or scarlet R did not, however, possess any sensitizing effect on syphilitic antigens, whereas water-soluble Victoria blue and Janus green were good sensitizers. It appears probable that sensitization by dyes takes place as a result of a chemical combination of the reacting substance of the alcoholic heart extract with the dye. In favour of this assumption is also the fact

that both reacting substance and dye appear in the precipitate, and that one substance cannot be separated from the other by washing with saline solution.

One of the distinctive features of the sensitizing action of dyes is the fact that in their presence much smaller concentrations of electrolytes are sufficient for precipitation than in their absence. The mechanism of the sensitizing action of dyes, however, differs from the flocculating action of electrolytes. Sensitization of negatively charged antigen suspensions could be obtained not only by the positively charged Victoria blue or Janus green, but also by the negatively charged isamine blue. A further difference between the action of electrolytes and that of dyes is the fact that the former can sensitize the dispersed antigen suspensions, whereas the latter have to be present before dispersion for sensitization to take place.

There is a point of similarity between the sensitizing action of dyes and the action of antibody on antigen. Both antigens sensitized by dyes and antigens sensitized by homologous antibody possess an increased sensitivity to the flocculating action of electrolytes. The sensitizing action of the dye may be pictured as an antibody-like effect which has been deprived of its specificity, and which is conditioned by the presence of a specific factor in syphilitic serum. Dean (1937) also observed that isamine blue precipitates globulin under suitable conditions, and that the process resembles an antigen-antibody reaction in respect of optimum proportions. Marrack (1940), when studying precipitation of horse serum euglobulin by isamine blue, also concluded that the reaction resembles the precipitation of antibody to pneumococci by the specific polysaccharide in several respects. The study of similar systems, as has been already pointed out by Marrack, and particularly the study of the mechanism of the sensitizing action of certain dyes, may help to elucidate the reaction between antigen and antibody.

The Dye Test for Syphilis.

Making use of the sensitizing action of certain dyes, a practical test for the serological diagnosis of syphilis was devised. Sensitization by dyes gave the test peculiar advantages of its own, namely, the ease and exactitude with which the sensitivity and reliability of the test could be standardized and maintained at a desired level. The sensitivity and specificity of an antigen for serological tests for syphilis, when not containing dyes, is influenced very markedly by ageing or ripening, i.e. the time allowed to pass between dispersion of the antigen and its use in the test. Standardization of the sensitivity of the test becomes difficult, as ageing is a continuous process. In the presence of a suitable dye, however, the test can not only be adjusted to a desired sensitivity by alterations in the dye content of the antigen, but the antigen suspension can also be rendered more stable. It will possess its full sensitivity immediately after dispersion, and the same level of sensitivity will be maintained for some time thereafter.

Very fine dispersion of the alcoholic antigen was obtained in the presence of Victoria blue. Therefore this dye was used for sensitization in the test in preference of Janus green. Many different alcoholic heart extracts and fractions therefrom were found equally amenable to sensitization by dyes. Kahn's extract is recommended because it is easily prepared and also obtainable commercially.

Preparation of the stock antigen.—To 10 parts of Kahn's standard antigen containing 0·6 per cent. of cholesterol and having a titre of 1 + 1·2, add 1 part of tinct. benz. co. B.P. To 10 ml. of this mixture add 17 mg. of Victoria blue 4R (Gurr). This stock antigen keeps at least for a year and probably indefinitely. It must be kept well stoppered, and care taken to prevent evaporation during preparation and handling.

Should an antigen of greater or lesser sensitivity be desired, the amount of dye may be increased or decreased. The use of antigens of greater sensitivity is, however, not recommended, as further increase of sensitivity is not possible without a decrease in the specificity of the reaction.

Dispersion of the antigen.—One part of the stock antigen, say, 0·1 ml., was pipetted to the bottom of a serological tube, and 10 parts (1 ml.) of 0·85 per cent. sodium chloride solution were blown rapidly into the tube from a pipette having a large opening at the tip. One ml. of dispersed antigen was sufficient for the performance of at least 30 tests. The antigen suspension could be used at once after dispersion, or at any time on the day of dispersion. It should be shaken immediately before use.

Technique of the test.—Sera to be tested were heated in a water bath at 56° C. for 30 minutes or at 62° C. for 4 minutes. One drop of heated serum was put on a glass slide and a drop of equal size of the dispersed antigen added. The drops were mixed with a glass rod and the slide rocked for 4 minutes. For convenience a large 4 by 6 in. glass slide, which was divided into 20 compartments by glass ink, was used. To read the results of the test the slide was placed on a sheet of white paper, and if the reaction was positive, large, deep blue floccules were easily visible in a water-clear fluid. If the reaction was negative the drop remained homogeneous and of sky blue colour. To detect the presence of small blue clumps in sera giving doubtful results, one end of the slide was lifted an inch or so above the paper. The results were read with the naked eye.

Comparison of the dye test with the Wassermann and Kahn tests.

The sensitivity and specificity of the dye test was compared with that of Wyler's (1929) modification of the Wassermann reaction and Kahn's (1928) standard test on 1112 sera. Table III gives a summary of the results of the three tests. In all, 187 positive and doubtful sera were detected by the combined use of the three tests. The table shows that the dye test was of greater sensitivity than either of the other tests. There were 26 sera reacting in the dye test but giving a negative result in the Wassermann and Kahn tests. The clinical histories of the patients whose sera reacted only in the dye test were investigated, and it was found that 5 were cases of early untreated syphilis, 3 cases of late untreated syphilis, 15 were cases which were under treatment for syphilis or had treatment for the disease at a previous date, 1 was a case of general paralysis of the insane, and in 2 cases information as to the clinical condition of the patient could not be obtained. The result of this inquiry showed that the results given by the dye test were specific.

TALBE III.—*Summary of Results Obtained with* 1112 *Sera Examined by the Wassermann, Kahn and Dye Tests.*

Test.	Number of positive results.	Number of doubtful results.	Number of negative results.
Wassermann	131	20	961
Kahn	124	19	969
Dye	160	18	934

Although the dye test is somewhat more sensitive than Wyler's modification of the Wassermann test or Kahn's flocculation test, it was not as sensitive as the collodion particles agglutination test (Berger, 1943). The dye test is, however, easier to perform, as very little laboratory equipment is required for its performance and the stock antigen keeps indefinitely.

DISCUSSION.

Addition of various dyes to syphilitic antigens has been recommended by numerous authors. The sensitizing action of certain of these dyes seems, however, to have been overlooked. Borowskaja (1927–28) recommended addition of Victoria blue to the antigen for Meinicke's turbidity test in order to facilitate reading of the results. She noted the indicator effect of the dye, but did not notice its sensitizing effect. Later, Meinicke (1934) added 0·01 g. of Victoria blue to 100 ml. of his antigen for the clarification test. Victoria blue in that concentration could not have exerted any sensitization, and Meinicke did not give his reason for the addition. Rosenthal (1929–30) added methylene blue to his antigen to make the final results more conspicuous for reading, and Márquez (1941) added acriflavine for similar reasons. Laughlen (1935), Levy (1941), Leiboff (1940), Lorenz (1940) and Rappaport and Eichhorn (1943) added fat-soluble Sudan III or scarlet R to their respective antigens. Ide and Ide (1936) coloured their antigen with crystal violet and azur II.

Sensitization by dyes does not seem to be confined to reactions for syphilis. It may be possible to find suitable dyes which would sensitize various antigen-antibody reactions. A few observations pointing in this direction have been recorded in the literature. Dean (1937) pointed out that addition of isamine blue to mixtures of horse serum and its antiserum produced a precipitate which was larger than that in a comparable mixture of antigen and antibody without dye. Dean also found that the use of isamine blue may extend the range of dilutions in which an antiserum could be used. Berger and Brecher (1939), when describing a slide test for the exclusion of typhoid fever, found that the addition of crystal violet to the suspension was of some importance for the sensitivity of the reaction. Other dyes which were tried did not possess the sensitizing action. Boyd (1939) observed that antisera for blood group factors, when treated with acriflavine or brilliant green, kept better than those simply kept sterile or treated with toluene. The dyes may have acted as sensitizer in these cases.

SUMMARY.

1. The influence of a number of different dyestuffs on the flocculation test for syphilis was investigated.

2. Janus green, Victoria blue and certain other dyes had both a sensitizing and indicator action on the reaction. Syphilitic sera containing too little of the reacting substance to react with a suitable heart extract without dye showed marked flocculation in the presence of Victoria blue and Janus green. These dyes were also taken up by the precipitate, leaving a water-clear supernatant fluid.

3. The sensitivity of the reaction was a function of the amount of dye. Antigens containing dyes attained their full sensitivity immediately after dispersion, and maintained the same level of sensitivity for some time thereafter.

4. Making use of the sensitizing effect of Victoria blue, a simple and reliable slide flocculation test for the serological diagnosis of syphilis was devised. This dye test was just as specific as, and somewhat more sensitive than, other commonly used serological tests for syphilis. The results of the test were easy to read.

5. The mechanism of the sensitizing action of dyes is discussed, and the possibility of sensitization of other antigen-antibody reactions by suitable dyes is pointed out.

This investigation was carried out at the instigation of Prof. P. L. Sutherland, to whom the author is indebted for much valuable criticism. The author also wishes to thank Prof. H. R. Dean for a sample of isamine blue.

REFERENCES.

BERGER, F. M.—(1943) *J. Path. Bact.*, **55**, 363.

Idem AND BRECHER, G.—(1939) *J. Hyg. Camb.*, **39**, 294.

BOROWSKAJA, D.—(1927–28) *Zbl. Bakt.*, **105**, 455.

BOYD, W. C.—(1939) *J. Immunol.*, **37**, 65.

BROWNING, C. H., AND MACKENZIE, I.—(1924) 'Recent Methods in the Diagnosis and Treatment of Syphilis.' London, 2nd ed.

DEAN, H. R.—(1937) *J. Path. Bact.*, **45**, 745.

EAGLE, H.—(1930) *J. exp. Med.*, **52**, 747.—(1931) *Ibid.*, **53**, 605.

IDE, S., and IDE, T.—(1936) *J. Lab. clin. Med.*, **21**, 1190.

KAHN, R. H.—(1928) 'The Newer Knowledge of Bacteriology and Immunology,' ed. by E. O. Jordan and I. S. Falk, Chicago, 848.

LAUGHLEN, G. F.—(1935) *Canad. med. Ass. J.*, **33**, 179.

LEIBOFF, S. L.—(1940) *J. Lab. clin. Med.*, **25**, 317.

LEVY, M. A.—(1941) *Am. J. clin. Path.*, **11** (*Technical Section*), 150.

LORENZ, W. F.—(1940) *Ibid.*, **10**, 527.

MÁRQUEZ, F.—(1941) *Am. J. Syph., Gon. and Ven. Dis.*, **25**, 319.

MARRACK, J. R.—(1940) *Proc. III Intern. Congr. Microbiol., New York*, 821.

MEINICKE, E.—(1934) *J. Lab. clin. Med.*, **19**, 518.

RAPPAPORT, F., AND EICHHORN, F.—(1943) *Lancet*, i, 426.

ROSENTHAL, L.—(1929–30) *Proc. Soc. exp. Biol. N.Y.*, **27**, 61.

WYLER, E. J.—(1929) 'Medical Research Council, Spec. Rep. Ser. no. 129.' London.

INDEX TO AUTHORS

BECK, S., see *Peacock, P. R.*

BERENBLUM, I., and SCHOENTAL, R., carcinogenic constituents of shale oil, 232

BERGER, F. M., the sensitizing and indicator action of Victoria blue and Janus green on the flocculation reaction for syphilis, 252

BERNKOPF, H., see *Kligler, I. J.*

CARR, J. G., prolonged antibody production following recovery of fowls from Rous No. 1 sarcoma, 138

——— some investigations upon the nature of the resistance of an inbred line of fowls to the development of the Rous No. 1 sarcoma, 127

——— the relation between age, structure, and agent content of Rous No. 1 sarcoma, 133

CHAIN, E., FLOREY, H. W., JENNINGS, M. A., and WILLIAMS, T. I., helvolic acid, an antibiotic produced by *Aspergillus fumigatus, mut. helvola* Yuill, 108

CROWFOOT, D. M., and LOW, B. W., a note on the crystallography of helvolic acid and the methyl ester of helvolic acid, 120

DANIELLI, J. F., see *Fell, H. B.*

DMOCHOWSKI, L., and GYE, W. E., influence of cross-suckling on the incidence of mammary cancer in high and low-cancer strains of mice, 223

DONIACH, I., MOTTRAM, J. C., and WEIGERT, F., the fluorescence of 3 : 4-benzpyrene *in vivo*.
I. The distribution of fluorescence at various sites, especially the skin of mice, 1
II. The inter-relationship of the derivatives formed in various sites, 9

ELLIOTT, S. D., type relationships amongst group A streptococci, 159

EVANS, D. G., the protective properties of the alpha antitoxin and theta antihaemolysin occurring in *Cl. welchii* type A antiserum, 81

FELL, H. B., and DANIELLI, J. F., the enzymes of healing wounds. I. The distribution of alkaline phosphomonoesterase in experimental wounds and burns in the rat, 196

FINDLAY, G. M., see *Maegraith, B. G.*

FLOREY, H. W., see *Chain, E.*

FULTON, F., staphylococcal enterotoxin, with special reference to the kitten test, 65

GRIESBACH, W. E., and PURVES, H. D., studies on experimental goitre. V. Pituitary function in relation to goitrogenesis and thyroidectomy, 174

——— ——— the assay of thyrotropic activity by the cell height response in guinea-pigs, 185

GYE, W. E., see *Dmochowski, L.*

HARRIS, G. C. M., see *Wilkins, W. H.*

JACKSON, E. B., see *Turner, J. C.*

JAPA, J., a study of the morphology and development of the megakaryocytes, 73

JENNINGS, M. A., see *Chain, E.*

KASSANIS, B., neutralization of some plant viruses by rabbit sera, 152

KEELE, C. A., see *Scarff, R. W.*

KLIGLER, I. J., and BERNKOPF, H., the path of dissemination of rabies virus in the body of normal and immunized mice, 15

LOW, B. W., see *Crowfoot, D. M.*

MCILWAIN, H., nutritional studies of bacterial variation.
I. Resistance to pantoyltaurine in naturally-occurring and experimentally prepared strains, 203
II. The derivation of drug-resistant strains in the absence of any inhibitor, 212

MCINTOSH, J., and SELBIE, F. R., the production of drug resistant cultures of bacteria *in vitro* and a study of their inter-relationships, 246

MACOUN, S. J. R., see *Prunty, F. T. G.*

MAEGRAITH, B. G., MARTIN, N. H., and FINDLAY, G. M., the mechanism of red blood cell destruction, 58

MARTIN, N. H., see *Maegraith, B. G.*

MERLING, K. B. E., ultramicroscopical observations on the morphology and development of vaccinia virus *in vitro*, 240

MILES, A. A., and MILES, E. M., the fixation of foreign material in inflamed tissue, with especial reference to the action of *Cl. welchii* toxin and antitoxin, 95

MILES, E. M., see *Miles, A. A.*

MORGAN, W. T. J., an artificial antigen with blood-group A specificity, 41

MOTTRAM, J. C., see *Doniach, I.*

OSBORN, E. M., on the occurrence of antibacterial substances in green plants, 227

PAGE, J. E., and ROBINSON, F. A., an examination of the relationship between the bacteriostatic activity and the normal reduction potentials of substituted quinones 89
PEACOCK, P. R., and BECK, S., multiple mesenteric sarcomata in rats following ingestion of heated lard, 143
PRUNTY, F. T. G., and MACOUN, S. J. R., the chloride excretion in steatorrhoea: comparison with the conditions in Addison's disease, 22
PURVES, H. D., studies on experimental goitre. IV. The effect of di-iodotyrosine and thyroxine on the goitrogenic action of brassica seeds, 171
——— see *Griesbach, W. E.*

RENNIE, J. B., a modified laevulose (fructose) tolerance test compared with the hippuric acid synthesis test of hepatic efficiency, 26
ROBINSON, F. A., see *Page, J. E.*
ROBSON, J. M., and SCOTT, G. I., the production and treatment of experimental pneumococcal hypopyon ulcers in the rabbit, 50

SCARFF, R. W., and KEELE, C. A., the effects of temporary occlusion of the renal circulation in the rabbit, 147
SCHOENTAL, R., see *Berenblum, I.*
SCOTT, G. I., see *Robson, J. M.*
SELBIE, F. R., viability of *Treponema pallidum* in stored plasma, 150
——— see *McIntosh, J.*
SIMPSON, R. E. H., criteria of life and death in human pus cells, 218
STUART-HARRIS, C. H., observations on the agglutination of fowl red cells by influenza viruses, 33

TENENBAUM, E., a pure strain of Rous sarcoma cells, 56
TURNER, J. C., and JACKSON, E. B., serological specificity of an autoantibody in atypical pneumonia, 121

WEIGERT, F., see *Doniach, I.*
WHITEHEAD, V. I. E., basal metabolism of the albino rat fed on goitrogenic diet, 192
WILKINS, W. H., and HARRIS, G. C. M., investigation into the production of bacteriostatic substances by fungi. Preliminary examination of a second 100 fungal species, 141
WILLIAMS, T. I., see *Chain, E.*

SURVEY OF PAPERS.

OSBORN has examined 2600 plants belonging to 166 families for substances inhibiting the growth of *Staphylococcus aureus* and *Bact. coli*. Of these, 63 genera contained substances which inhibited one or both test organisms (p. 227).

BERENBLUM AND SCHOENTAL, using a chromatographic absorption method, have separated carcinogenic blue shale oil into fractions with distinct banded fluorescent spectra. 3 : 4-benzpyrene is present in a concentration too low to account for more than a small part of the oil's carcinogenic action, whereas the most active fraction is entirely free from benzpyrene. Their results support the view that the carcinogenic constituents of blue shale oil are similar to those responsible for carcinogenic action in coal-tar, and that their presence is due to temperature effects during the process of its production from the shale (p. 232).

MERLING, in a continuation of his observations on slide cultures of vaccinia virus, now shows that the virus forms colonies which appear as cysts ; these move on the slide and divide to form daughter-cysts. Within these cysts all forms of the life-cycle of the virus can be seen. By the application of the author's methods for obtaining high magnification and resolution with visible light, the elementary bodies appear as oblongs (p. 240).

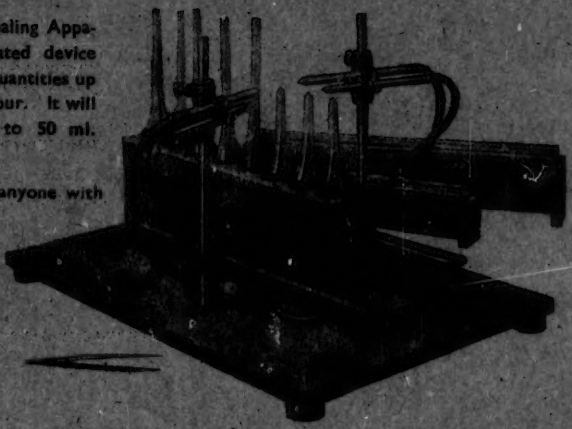
MCINTOSH AND SELBIE have produced streptococci resistant to sulphonamides and staphylococci resistant to propamidine and some of the acridine compounds by repeated subculture of the organism in increasing concentrations of the respective drugs. Cultures of staphylococci resistant to propamidine can be obtained with great facility, not only by exposure to the action of propamidine, but also by the action of some acridines, and are also resistant to some of the acridines. Sulphanilamide fast streptococci are also resistant to other sulphonamides with the exception of marfanil. Thus, drugs of similar chemical constitution may act in dissimilar ways, and again dissimilar drugs may act in similar ways. The method used affords a new approach to studies on the relation of chemotherapeutic action to bacterial metabolism (p. 246).

BERGER has studied the influence of dyestuffs on the flocculation test for syphilis. Janus green, Victoria blue and certain other dyes have both a sensitizing and indicator action, so that syphilitic sera containing too little of the reacting substance to give flocculation with a suitable heart extract show marked flocculation in the presence of these dyes. Making use of this effect he has devised a simple and reliable flocculation test for syphilis which is as specific as, and more sensitive than, other serological tests for syphilis (p. 252).

B·T·L AMPOULE SEALING APPARATUS

The B·T·L Ampoule Sealing Apparatus is a hand-operated device which will deal with quantities up to 300 ampoules per hour. It will handle ampoules up to 50 ml. capacity.

With a little practice anyone with no experience of glass-blowing can obtain clean rounded seals, and the length of the sealed ampoules is automatically regulated to within a millimetre or two.



BAIRD & TATLOCK (LONDON) LTD.
14–17 ST. CROSS STREET, LONDON, E.C.1

GURR'S
Standardized STAINS for Microscopy

are used in the principal Laboratories of the Empire

STAINS in Powder, Solution or Tablet Form, Mounting Media, Bacteriological Sugars, Immersion Oils, Medicinal Dyes, Indicators, Filtered Paraffin Wax, Microscopical Accessories, etc.

Price List and literature on application

GEORGE T. GURR
136 NEW KING'S ROAD
LONDON, S.W.6
Works: ELYSIUM PLACE S.W.6

Consult
PRESTCOLD
for any Temperature Control Problem

PRESTCOLD *Refrigeration*
LARGEST BRITISH MANUFACTURERS OF SPECIALISED REFRIGERATION EQUIPMENT
PRESSED STEEL CO. LTD. COWLEY · OXFORD

W. WATSON & SONS LTD.

Manufacturers of **MICROSCOPES** for All Purposes

and

ALLIED APPARATUS

for every form of Scientific Investigation, Manufacturing Control, etc.

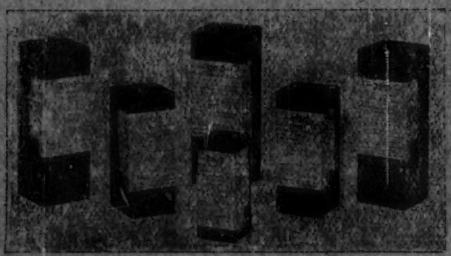
OPTICAL ELEMENTS & OPTICAL SYSTEMS

All Optical and Scientific Instruments

Temporary Head Office, *to which inquiries should be addressed—*

14, HADLEY GROVE, HIGH BARNET, HERTS

Cables: OPTICS, BARNET **Telephone: BARNET 2560**



DIFCO

DEHYDRATED CULTURE MEDIA AND REAGENTS

Prepared in the Laboratories, Detroit, Michigan, U.S.A.

DIFCO Products stand unsurpassed for ease and convenience in the preparation of culture media, whether in large or small quantities.

The process is economical and time-saving, all that is necessary being to weigh accurately the dehydrated medium, dissolve it in distilled water, and sterilize the resulting solution.

No adjustment of the reaction is needed, as the final reaction of the sterilized medium is shown on the label of each bottle, and further, no filtration of the liquid is required.

Complete list sent on application to Dept. AK/3

BAIRD & TATLOCK (LONDON) LTD.

14–17 ST. CROSS STREET, LONDON, E.C.1

PRINTED BY ADLARD AND SON, LIMITED, AT THEIR WORKS, BARTHOLOMEW PRESS, DORKING.